Nádia Filipa Marques Grilo

Licenciada em Biologia

Protein adducts from the anti-HIV drug abacavir – possible biomarkers of drug toxicity

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Sofia de Azeredo Pereira, Professora Auxiliar Convidada, Faculdade de Ciências Médicas, UNL
Co-orientador: Alexandra Moita Antunes, Investigadora Auxiliar, Instituto Superior Técnico, UTL

Jurí:

Presidente: Prof. Doutora Ilda Sanches
Arguente: Prof. Doutor Frederico Pereira
Vogal: Prof. Doutora Sofia Pereira

FACULDADE DE CIÊNCIAS E TECNOLOGIA
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Caixas U, Ferreira J, Marinho AT, Faustino I, **Grilo NM**, Lampreia F, Germano I, Monteiro EC, Pereira SA. 2012. Longterm maraviroc use as salvage therapy in HIV-2 infection. J Antimicrob Chemother 67: 2538-2539.

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Abstract

While the benefits of combined antiretroviral therapy have revolutionized the life expectancy of patients infected with the human immunodeficiency virus, treatment-associated toxicity is frequently observed. Abacavir is a nucleoside reverse transcriptase inhibitor associated with acute toxic events, such as hypersensitivity reactions. In addition, its long-term use has increasingly been recognized as associated with an increased risk of myocardial infarction. While the mechanisms underlying abacavir-induced hypersensitivity and cardiotoxicity are not fully understood, abacavir bioactivation to a reactive aldehyde metabolite is thought to play a crucial role in this context. However, as a short lived specie, *in vivo,* its formation has so far eluded detection. Our initial hypothesis was that it could be efficiently trapped by *N*-terminal valine of hemoglobin, forming *N*-terminal-valine-abacavir adducts.

With the ultimate goal of gain insight into the role of abacavir metabolism in abacavir-induced toxicity, the present work was focused on obtaining evidence for the abacavir bioactivation to a reactive aldehyde metabolite, in human immunodeficiency virus - infected patients, and on the evaluation of this reactive metabolite ability to undergo protein modification. To address these issues: 1) abacavir-*Edman* standards were prepared to monitor the presence of these adducts *in vivo*; 2) the presence of abacavir-*Edman* adduct was screened in *Wistar* rats, for optimization of analytical method; and 3) in patients infected with the human immunodeficiency virus. The experimental approach used, involving *N*-Alkyl *Edman* degradation, for specific detachment of *N*-terminal-valine-abacavir adducts from protein, followed by liquid chromatography-electrospray ionization tandem mass spectrometry analysis of detached abacavir-*Edman* adducts, upon comparison with standards prepared *in vitro*, allowed the unequivocal identification of these adducts in animal and human samples. These results represent the first report on the involvement of a conjugated aldehyde intermediate in the metabolic activation of abacavir in man. Whereas this evidence does not imply an exclusive relation between abacavir-*Edman* adducts and abacavir toxicity, the search for causal relationships between the formation of abacabir-derived protein adducts and the occurrence of abacavir-induced toxic events in patients is worth pursuing, and is currently underway, towards the clarification of mechanism(s) of abacavir-induced toxicity.

Keywords: Abacavir, aldehyde metabolite, biomarker, cardiotoxicity, drug bioactivation, hypersensitivity.

Resumo

Apesar da terapêutica antiretroviral combinada ter revolucionado a esperança de vida de doentes infectados pelo vírus da imunodeficiência humano, a toxicidade associada ao tratamento é frequentemente observada. O abacavir é um nucleósido inibidor da transcriptase reversa que está relacionado com eventos tóxicos agudos, tais como reacções de hipersensibilidade. A sua utilização a longo prazo tem sido também associada a um aumento do risco de enfarte do miocárdio. Apesar dos mecanismos subjacentes à hipersensibilidade e à cardiotoxicidade induzidas pelo abacavir não serem completamente compreendidos. A bioactivação do fármaco a um metabolito aldeídico reactivo tem sido frequentemente apontada como tendo um papel crucial neste contexto. No entanto, a detecção deste metabolito *in vivo* ainda não foi conseguida, provavelmente devido ao curto tempo de vida deste tipo de metabolitos em condições fisiológicas. Como hipótese inicial, equacionou-se que este intermediário poderia ser armadilhado pela valina *N*-terminal da hemoglobina, formando um aduto covalente passível ser identificado/quantificado.

Tendo como objectivo global a elucidação do papel do metabolismo do abacavir na toxicidade induzida por este fármaco, o presente estudo visou a obtenção de evidencias para a ativação do abacavir a um aldeído reativo, em doentes infectados pelo vírus da imunodeficiência humano, e a avaliação do potencial deste metabolito reactivo para modificar covalentemente proteínas. Para alcançar estes objectivos:1) foram preparados sinteticamente padrões dos adutos de abacavir para monitorar a presença destes *in vivo*; 2) foi investigada a presença destes adutos em ratos Wistar expostos ao abacavir, com o vista à optimização da técnica analítica para a identificação/quanticação de adutos; 3) foi investigada a presença dos adutos em doentes. Através de um procedimento experimental que envolveu a libertação selectiva do aduto, por degradação de *Edman* da valina *N*-terminal, seguida da análise do aduto libertado por cromatografia líquida acoplada a espectrometria de massa com ionização por electrospray, e comparando com os padrões, foi possível identificar inequivocamente estes adutos *in vivo.* Estes resultados representam a primeira evidência do envolvimento de um aldeído conjugado na activação metabólica do abacavir no homem. Embora estes resultados não impliquem uma relação exclusiva entre a formação destes adutos e a toxicidade induzida pelo abacavir, sublinham a importância do estabelecimento de correlações entre a presença/concentração destes adutos e a ocorrência de eventos tóxicos. Atualmente, mais trabalho está a ser desenvolvido com o objectivo de esclarecer os mecanismos subjacentes à toxicidade do fármaco.

Palavras-chave: Abacavir, bioativação de fármacos, biomarcadores, cardiotoxicidade, hipersensibilidade, metabolito aldeídico.

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Abbreviations

ABC	Abacavir
ABC-MP	Abacavir monophosphate
ADH	Alcohol dehydrogenase
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen-presenting cell
BID	Twice daily
cART	Combination antiretroviral therapy
CBV-DP	Carbovir diphosphate
CBV-MP	Carbovir monophosphate
CBV-TP	Carbovir triphosphate
dGTP	Deoxyguanosine triphosphate
DMSO	Dimethyl sulfoxide
DMF	*N,N*-dimethylformamide
DNA	Deoxyribonucleic acid
FDA	Food and Drug Administration
Hb	Hemoglobin
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HSR	Hypersensitivity reactions
INI	Integrase Inhibitor
LC-ESI-MS/MS	Liquid Chomatography - Electrospay Ionization-Tandem Mass Spectrometry
MHC	Major histocompatibility complex
MI	Myocardial infarction
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleoside reverse transcriptase inhibitors
NtRTI	Nucleotide reverse transcriptase inhibitors
NVP	Nevirapine
OD	Once daily
PI	Protease inhibitor
PTIC	Phenyl isothiocyanate
RNA	Ribonucleic acid
TAP	Transporter associated with antigen processing
TCR	T-cell receptors
THF	Tetrahydrofuran

Abbreviations

TLC	Thin layer chromatography
3TC	Lamivudine

1. Introduction

1.1 Human Immunodeficiency Virus Infection and the combined antiretroviral therapy

More than thirty years have passed since human immunodeficiency virus (HIV)-infection epidemic began. However, this infection is still a serious worldwide public health problem. Nevertheless, the success of the combined antiretroviral therapy (cART) changed dramatically the prognosis of this infection, which is currently perceived as a chronic disease, particularly in the developed countries (Mehellou and De Clercq, 2010). At the end of 2010, 6.65 million people were on cART and this number is still increasing (WHO *et al.*, 2011). Despite its indisputable benefits, cART life-long use raises concerns about its adverse effects, especially the long-term toxic effects. This potential toxicity of antiretroviral drugs can have a negative impact on clinical outcomes, ultimately affecting the quality of life and life expectancy of the patients. Moreover, the management of toxicity outcomes may require additional hospital visits and admissions, increasing the economic burden on already strained medical care systems. Thus, an understanding of the mechanisms underlying drug toxicity is essential for establishing dependable risk-benefit relationships that can guide decisions on treatment options.

The HIV-infection can be considered as having four main stages. Following the primary infection, there is a clinical latent period, asymptomatic, with active viral replication associated with a progressive destruction of the CD4 cells. This stage can last an average of 8-11 years, depending on several factors including the person´s health status and life style. In this period of latency, there are enough immune cells to afford an immune response against the infection. But, eventually, a significant number of T cells are destroyed and the rate of production of these cells cannot follow the rate of its destruction, and the patient comes to a symptomatic stage. The cART is recommended to begin in this stage, according to the national (DGS, 2012) and international guidelines (EACS, 2011), when CD4 count is bellow 350 cell/mm^3. CD4 counts should be obtained every 3-6 months during periods of clinical stability, and more frequently should symptomatic disease occur. If CD4 count drops to baseline or below 50% of increase from pre-treatment (aprox. 30 cells.mm^{-3}) then the cART should be changed. The maintenance of sequential viral load values < 150,000 copies/ml is associated with higher short-term survival rates, thus higher viral load is also a criteria to start the cART. Viral load is currently the preferred method for monitoring the response to cART. There should be at least 1 log reduction in viral load, preferably to less than 10,000 copies/ml HIV-RNA within 2-4 weeks after the beginning of treatment. If a lower reduction in viral load is observed or it stays above 100,000 cps/mL, then the treatment should be adjusted by either adding or switching drugs. After six months it should be maintained lower 20 cps/mL. Viral load measurement should be repeated every 4-6 months if patient is clinically stable. If viral load returns to 0.3-0.5 log of pre-treatment levels, or there are two consecutive blips in its value, then the cART is no longer working and should be changed. The appearance of AIDS-defining conditions and the deterioration of the immune system causes the progression of the HIV-infection to AIDS.

The first-line cART comprises different alternatives adapted to each patient. First line cART is always composed by three drugs, two of which are nucleoside or nucleotide reverse transcriptase

inhibitors (NtRTI; tenofovir/emtricitabine or abacavir/lamivudine) plus one of the following options a non-nucleoside reverse transcriptase inhibitor (NNRTI; efavirenz or nevirapine); one protease inhibitor (PI; atazanavir or darunavir) boosted with ritonavir; one integrase inhibitor (INI, raltegravir). (EACS, 2011; DGS, 2012). Occasionally, fist-line regimens needs to be altered - for example in case of virological failure, side effects, pregnancy, co-infections - and alternative regiments are adopted (EACS, 2011; DGS, 2012).

As a cure to the HIV infection seems not likely to occur in a near future, chronic treatment with anti-HIV drugs to control the infection is presently unavoidable. Therefore, the greater the knowledge about the risks associated with these drugs; greater will be the support for the selection of the best drug for the right patient.

1.2 Antiretroviral drugs

Antiretroviral drugs are distributed by seven classes, according to their mechanism of action in the several steps of HIV life cycle (**Fig. 1.1**). The NRTIs, as abacavir (ABC), act by inhibiting the HIV reverse transcriptase enzyme. They are analogues of the naturally occurring deoxynucleotides and must be phosphorylated to their pharmacologicaly triphosphate derivates, to later compete with the natural deoxyribonucleotides for the incorporation into the growing DNA by cell DNA polymerases. Unlike the natural deoxyribonucleotides, NRTI do not have a 3'-hydroxyl group on the deoxyribose moiety and, as consequence, following incorporation, the next incoming deoxyribonucleotide cannot form the 5'-3' phosphodiester bond, which is needed to extend the DNA chain, leading to chain termination. The nucleotide reverse transcriptase inhibitors (NtRTIs), as tenofovir, are also pro-drugs and share with NRTIs the same mechanism of action. The difference between both classes is that NtRTs already have one phosphate group and only two additional phosphorylation steps are required to be converted into their pharmacological active form (Anderson *et al.*, 2004; Piliero, 2004). The NNRTIs, like efavirenz, also inhibit the reverse transcriptase, but in a non-competitive manner. They bind to an allosteric site, near the active site of reverse transcriptase. This binding, changes the conformation of reverse transcriptase, distorting the position of the residues which bind DNA,inhibiting its polymerization (De Clercq, 2004). The protease inhibitors (PIs) prevent viral replication by inhibiting HIV protease, which is the responsible for cleavage precursor polyproteins to structural proteins (*eg.* p24) and functional proteins (*eg.* reverse transcriptase), preventing the formation of infectious virus particles. The only available fusion inhibitor (FI), T-20, acts at the interaction between viral gp120/gp41 and the receptors of the host cell. After the interaction between gp 120 and CD4 receptor and the conformational changes caused by them, the FI bind to gp 41 and prevents the fusion of HIV with the host cell membrane. The co-receptors antagonists, specifically the CCR5 receptor antagonists as maraviroc, bind to CCR5, blocking the biding of gp120 to the receptor and thus, blocking the binding of the virus to the host cell. Lastly, the integrase inhibitors (INIs), as raltegravir inhibit the integrase, which is the viral enzyme responsible for the integration of the viral DNA into the host cell genome (De Clercq, 2009; Hartman and Buckheit, 2012).

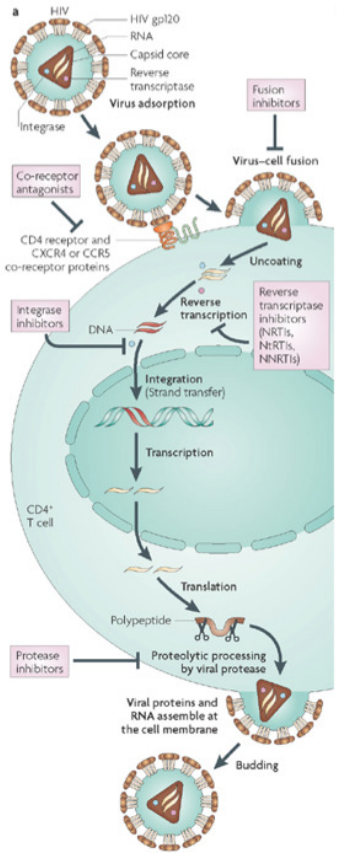

Figure 1.1. Schematic overview of the human immunodefiency virus (HIV)-life cycle main steps and the targets for antiretroviral drugs. Mechanism of action for the different classes of antiviral drugs nucleoside reverse transcriptase inhibitors (NRTIs), nucleotide reverse transcriptase inhibitors (NtRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), fusion inhibitors, protease inhibitors, co-receptor antagonists and integrase inhibitors throughout the different phases of HIV-life cycle. In: De Clercq (2007).

Despite the irrefutable benefits brought by cART, it is associated with several complications, as the increased prevalence of drug-resistant virus strains, the increased incidence of transmission of drug-resistant virus and the drug toxicity. For these reasons, the research for new compounds, new drug targets and novel therapeutic strategies, such as minimizing antiretroviral toxicity, are essential towards successful and safer treatment.

1.3 A focus on abacavir pharmacology

The NRTI ABC (**Fig. 1.2**) is a 2'-deoxyguanosine nucleoside analogue. This drug is commercially available since 1999, as ABC sulfate (Ziagen®), as part of a two-drug pill (ABC/lamivudine (3TC): Kivexa®) and as part of a three-drug pill (ABC/3TC/zidovudine: Trizivir®), for both adults and children, with anti-HIV type-1 or type-2 activities (Daluge *et al.*, 1997; Saag *et al.*, 1998; Hervey and Perry,

2000). The recommended ABC dosage for adults is 300 mg twice daily (BID) or 600 mg once daily (OD). In Kivexa® formulation, ABC is co-formulated with 3TC in a dose of 600 mg for ABC and 300 mg for 3TC allowing an OD schedule. However, in those patients with renal insufficiency 3TC dose adjustment is required and a BID schedule is recommended.

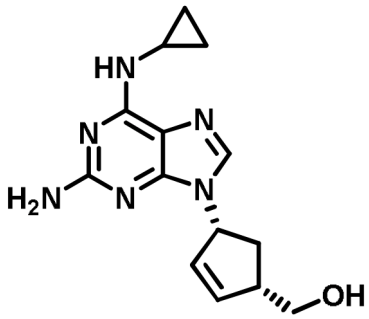

Figure 1.2. Structure of abacavir.

ABC enters cells by non-facilitated diffusion and binds o cytosolic proteins. The pharmacologic effect of ABC is achieved after its intracellular conversion to the carbovir thriphosphate (CBV-TP) metabolite via a stepwise anabolism, involving enzymes that are not implicated in the phosphorylation of the other NRTIs (Faletto *et al.*, 1997). Firstly, ABC is phosphorylated to abacavir 5'-monophosphate (ABC-MP) by adenosine phosphotransferase, followed by deamination *via* a cytosolic enzyme to form carbovir 5'-monophosphate (CBV-MP). Following subsequent phosphorylations, carbovir diphosphate (CBV-DP) and CBV-TP are generated, via guanylate kinase and nucleoside diphosphate kinase activities, respectively. The pharmacologically active metabolite CBV-TP competes with the endogenous 2'-deoxyguanosine triphosphate (dGTP) for the incorporation into the nucleic acid chain and, after incorporation, terminates DNA chain extension (**Fig. 1.3**) (Hervey and Perry, 2000; Yuen *et al.*, 2008).

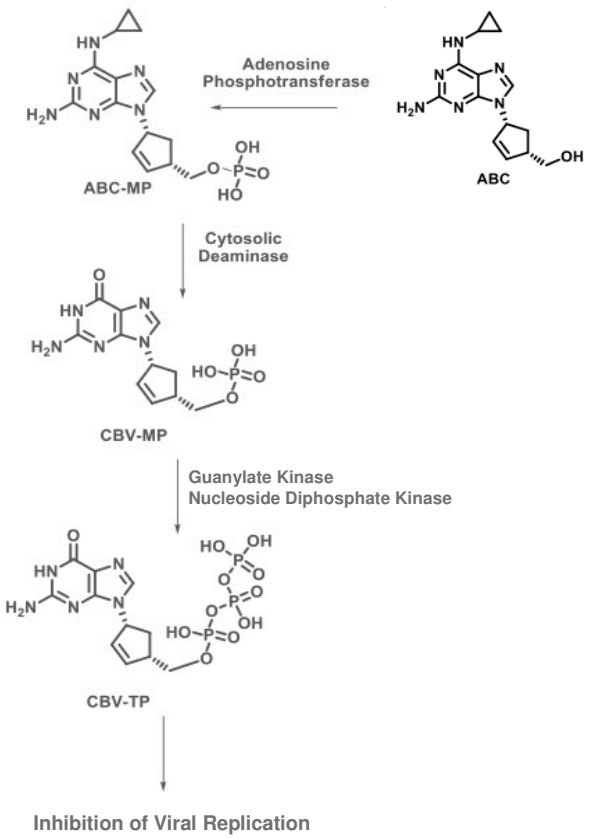

Figure 1.3. Intracellular conversion of abacavir (ABC) to its pharmacologically active metabolite carbovir triphosphate. Phosphorylation steps involve adenosine phosphotransferase, guanylate kinase nucleoside diphosphate kinase activities to generate abacavir monophosphate (ABC-MP), carbovir monophosphate (CBV-MP) and carbovir triphosphate (CBV-TP). This pharmacologically active metabolite, CBV-TP, competes with the endogenous 2'-deoxyguanosine triphosphate for the incorporation into the nucleic acid chain.

ABC absolute bioavailability in adults is about 83% (EMEA, 2010; Sivasubramanian *et al*., 2010). Maximum plasma concentration is reached in approximately 0.8 to 1 hour post-dose and ranges from 4.10 to 5.46 ìg/mL after single 600 mg dose and 2.39 to 11 ìg/mL after a single 300 mg dose (Chittick *et al*., 1999; McDowell *et al*., 1999; Yuen *et al*., 2001). This NRTI has a widespread penetration into body tissues, including the blood-brain-barrier, reaching the brain, which is a reservoir of HIV-infection. This NRTI can also cross the human placenta, allowing concentrations in the newborn at birth, which provides protection against HIV-transmission (Best *et al*., 2006). Unlikely the other drugs of the same class, ABC is extensively biotransformed by the liver, with less than 2% being eliminated unchanged in the urine (Chittick *et al*., 1999; McDowell *et al*., 1999). ABC is biotransformed *via* two pathways comprising a Phase II glucuronidation, mediated by uridine diphosphate glucuronyltransferase (UGT), yielding an inactive glucuronide metabolite; and a Phase I oxidation, mediated by alcohol dehydrogenase (ADH), yielding an inactive carboxylate metabolite. These metabolites are excreted by the kidney, where in combination they account for approximately 66% of the net dose; an additional 15% of the dose is converted into a number of minor metabolites (McDowell *et al*., 1999; Hervey and

Perry, 2000; Yuen *et al.*, 2008). ABC metabolites and unchanged ABC account for 83% of the administrated dose eliminated in the urine and the remainder is eliminated in the faeces.

It is well established that ABC can be bioactivated during the formation of the carboxylate metabolite (Walsh *et al.*, 2002). The formation of this metabolite involves a two-step oxidation process, *via* a reactive aldehyde intermediate. The formation of isomers the acid metabolite and the ABC in itself through ADH activity led Walsh *et al.* (2002) to propose a metabolic pathway involving double bond migration and epimerization processes upon formation of two putative aldehyde intermediates: an unconjugated aldehyde, which rapidly isomerizes into a thermodynamically more stable conjugated aldehyde (ABC aldehyde; **Fig. 1.4**). This conjugated ABC aldehyde is capable of reacting with proteins *in vitro* (Charneira *et al.*, 2011), generating adducts; which potentially can act as antigens and be recognized by the immune system, triggering an immune response (Park *et al.*, 2011a). However, so far this reactive metabolite has eluded detection in humans.

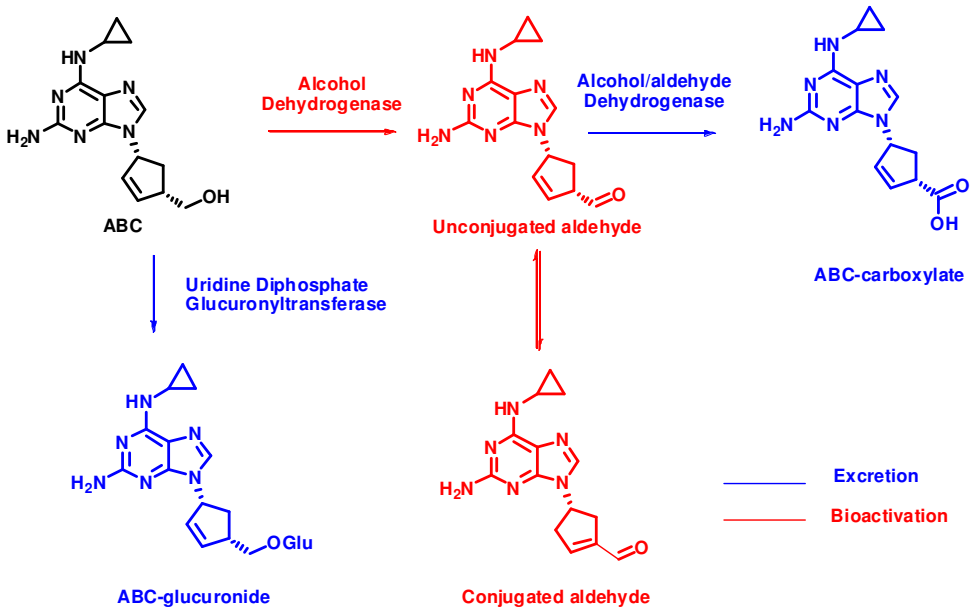

Figure 1.4. Scheme of abacavir bioactivation and elimination pathways. Metabolic pathway of abacavir (ABC) involving the formation of two putative aldehyde intermediates: the unconjugated aldehyde, which rapidly isomerizes into the conjugated aldehyde. This conjugated aldehyde is capable of reacting with proteins forming protein adducts (Charneira *et al.*, 2011).

1.4 A briefly overview on abacavir toxicity mechanism

Despite the therapeutic benefits of ABC in HIV-infection treatment, this antiretroviral has been associated with several adverse reactions. Nausea is the most frequently reported. In addition, vomiting, malaise and fatigue, headache, diarrhea, sleep disorders, cough, anorexia and rash have also been described, but in lower frequency. These adverse events tend to be more frequent soon after the starting of ABC and are mild or moderate in intensity and transient. The most concerning

factor of ABC use is its association with hypersensitivity reactions (HSR) and with myocardial infarction (MI).

1.4.1 Hypersensitivity reactions

HSR has been reported in 3 to 5% of patients, usually occurring within the first 6 weeks of ABC use (Clay, 2002) and can lead to drug discontinuation. This adverse reaction is characterized by symptoms representative of a multi-organ involvement. It is diagnosed by the manifestation of, at least, two of the following symptoms: rash, fever, vomiting, headache, respiratory and gastrointestinal symptoms, lethargy, myalgia or arthralgia (Mallal *et al.*, 2002). Usually, these symptoms rapidly disappear, after ABC discontinuation. Some patients can be misdiagnosed with respiratory diseases, gastroenteritis or reactions to other medications, which may result in a more severe reaction, multi-organ failure and death. The discontinuation of ABC must be immediate and cannot be re-challenged.

A strong association between ABC-induced HSR risk and a HLA-B*5701 allele has been described in several clinical trials. In the *Clinical Trial 1* (PREDICT-1) trial (Mallal *et al.*, 2008) and the *Study of Hypersensitivity to Abacavir and Pharmacogenetic Evaluation* (SHAPE) study (Saag *et al.*, 2008) it has been showed that those patients (especially among Caucasians) who carry the HLA-B*5701 allele were at higher risk for experiencing an ABC-induced HSR, with a positive predictive value of 47.9% and a negative predictive value of 100%. Therefore, current guidelines recommended the performance of a prospective test to detect the allele-positive patients, to identify to whom ABC should not be prescribe (Nolan, 2009). This prospective test is one of the major successes in the area of personalized medicine by mean of pharmacogenetics. However, this test does not predict which patients will definitely develop HSR (Mallal *et al.*, 2008), allowing the possibility of different mechanisms at the onset of HSR.

Currently, three major complementary models are considered for these immune-mediated adverse reactions: 1) the hapten/prohapten hypothesis (**Fig. 1.5**) (Uetrecht, 2007); 2) the pharmacologic interaction with immune receptors, the p-i hypothesis (**Fig. 1.6**) (Pichler *et al.*, 2006); and 3) the danger model, which is complementar of the hapten hypothesis, adding a secondary signal (eg. an infection) (**Fig. 1.7**) (Pirmohamed *et al.*, 2002). The hapten hypothesis considers that drugs or their metabolites are too small to be immunogenic. Instead, they can bind irreversibly to proteins, generating covalent adducts, which act as antigens (Uetrecht, 2007). These drug-modified proteins are seen as foreign by the immune system, leading to an immune response. Possibly, theses adducts are processed by antigen presenting cells (APCs) into peptide fragments, which associate with the major histocompatibility complex (MHC) from class I (genes *HLA-A*, *-B* and *–C*) and are presented to T-cell receptors (TCR) (Uetrecht, 2007; Bharadwaj *et al.*, 2010; Park *et al.*, 2011b). The p-i hypothesis advocates a non-covalent interaction between the drug and the MHC complex and/or the TCR, without the involvement of a specific peptide. The danger hypothesis argues that in addition to a signal 1 (the biding of the drug or drug-protein adduct to MHC complex), a signal 2 (eg. from injured cells, such as those exposed to pathogens or toxins) it is also necessary for activate an immune response. Without this signal the response is tolerance to signal 1 (Pirmohamed *et al.*, 2002; Uetrecht, 2007).

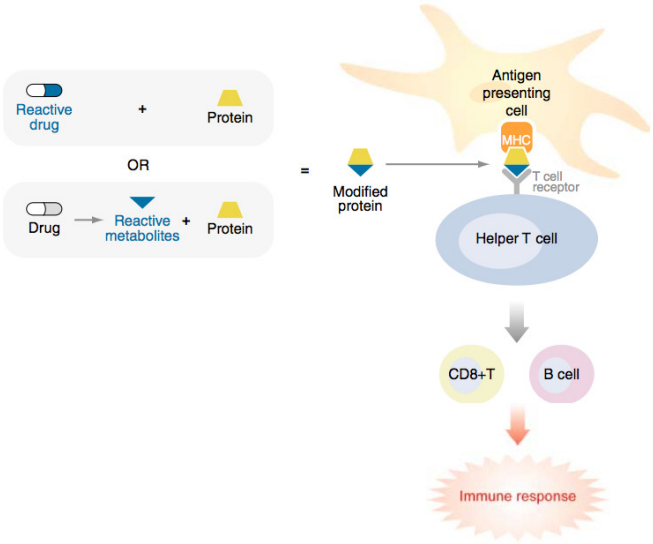

Figure 1.5. Hapten/prohapten hypothesis. The drug or reactive metabolite covalently binds to protein. The modified protein is taken up by antigen presenting cells, processed, and presented through major histocompatibility complex (MHC) to helper (CD4) T cells. In: Uetrecht (2007)

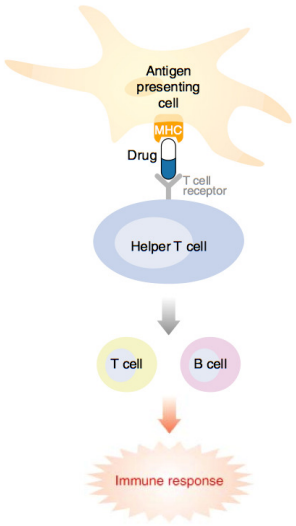

Figure 1.6. Pharmacological interaction (P-i) hypothesis. The drug binds directly to the major histocompatibility complex leading to an immune response to the parent drug. In: Uetrecht (2007).

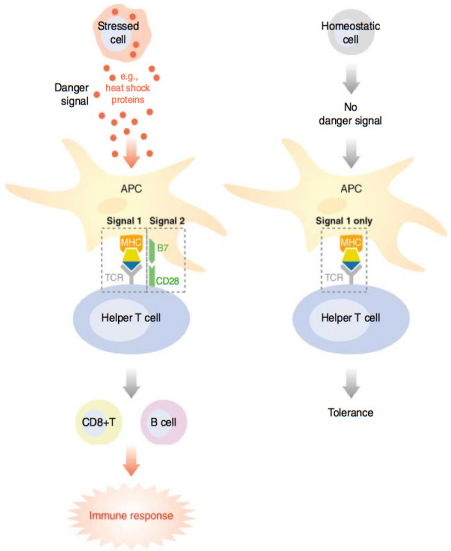

Figure 1.7. Danger hypothesis. Together with the signal from the hapten hypothesis is also necessary a signal 2, which will lead to a co-stimulation of T-cells. In: Uetrecht (2007).

Additionally, a specific model for restricted generation of immunogenic complexes in ABC-induced HSR, through the hapten hypothesis, was recently suggested (Bharadwaj *et al.*, 2012), taking into account the link to the HLA-B*57:01. Briefly, the covalent modification of a protein, by ABC or a metabolite, generates a hapten. This hapten is processed in the cytoplasm by proteasome to produce peptide fragments, which are transferred into the endoplasmic reticulum by the transporter associated with antigen processing (TAP) (McCluskey *et al.*, 2004), where peptide loading is optimized by tapasin (Williams *et al.*, 2002b). Then the peptides are loaded onto HLA-B*5701. The antigen-specific T cells recognizing the HLA-B*5701-restricted viral peptide, causing an immune response (**Fig. 1.8).** Functional loss of either TAP or tapasin leads to defective peptide loading and impaired antigen presentation (Williams *et al.*, 2002a).

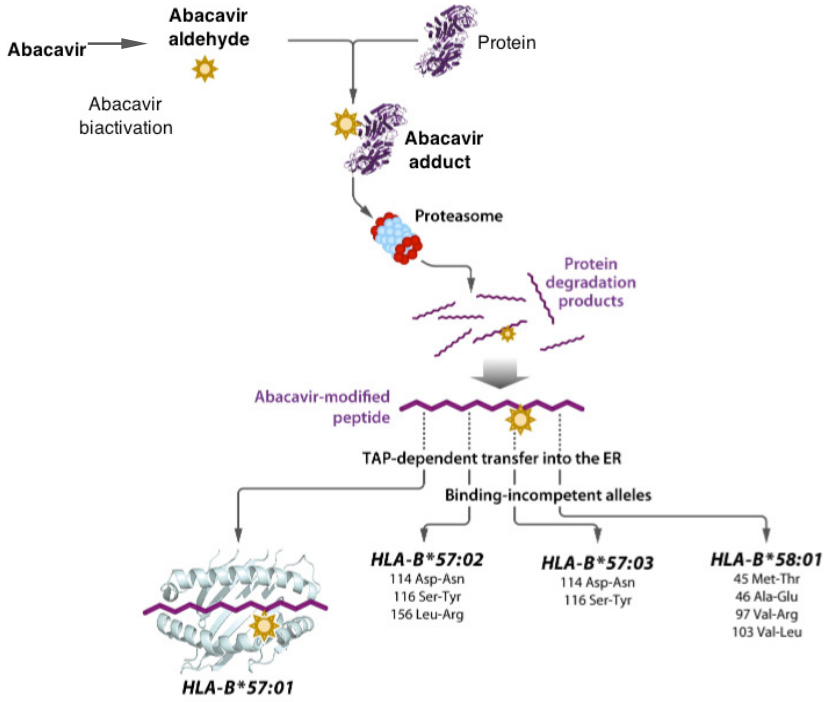

Figure 1.8. Hapten hypothesis for a restricted generation of immunogenic complexes in abacavir hypersensitivity syndrome on HLA-B*57:01 positive patients. Adapted from Bharadwaj *el al.* (2012).

1.4.2 Heart toxicity mechanism

In addition to the adverse effects described above, long-term ABC exposure has been associated with an increased risk of MI (Costagliola *et al.*, 2010; Worm *et al.*, 2010; Islam *et al.*, 2012). This association is still controversial, but a recent meta-analysis (Islam *et al.*, 2012) report that together with the fact that HIV-patients are at higher risk of devolving cardiovascular disease, ABC is the antiretroviral drug associated with greater risk of MI. Also, current guidelines recommend caution with ABC administration in patients at higher risk of cardiovascular disease (Thompson *et al.*, 2010) and also FDA has been showing concern about this problem, when in March 2011 presented an alert for MI risk associated with ABC (FDA, 2011).

Based on well documented knowledge of the link between drug aldheyde metabolites and cardiotoxicity (Guo and Ren, 2010a), it can be anticipated that ABC aldehyde might play a significant role at the onset of ABC-induced heart toxic effects. Nevertheless, this ABC aldehyde has never been identified in man, probably due to the lack of suitable methodological approaches.

1.5 Reactive metabolites, protein-adducts and drug toxicity

The exact mechanism for both cardiotoxicity and HSR associated ABC is not clear. However, ABC bioactivation can be involved. The reactive metabolites generated from drug biotransformation are usually electrophile species, which react easily with macromolecules (eg. proteins), generating adducts. The nucleophilic sites of proteins such as cysteine thiols, lysine amines, histidine imidazoles side chains and protein *N*-terminal amines (Guengerich *et al.*, 2001; Casini *et al.*, 2002) are the expected targets of reaction with the electrophilic reactive metabolites.

Whereas solid proof for the involvement of ABC bioactivation in toxic events induced by this antiretroviral has not been found yet, some evidence support this hypothesis: 1) the formation of adducts has been implicated in several drug-induced toxic events (Levine and Ovary, 1961; Padovan *et al.*, 1996); 2) aldehydes are short-lived and extremely difficult to detect *in vivo*, but can form stable covalent adducts with proteins; 3) aldehydes are often implicated in HSR conditions due to their ability to undergo protein modification (**Table 1.1**) (O'Brien *et al.*, 2005); 4) ADH is present in epithelial tissues, including the skin (Lockley *et al.*, 2005) and heart (Estonius *et al.*, 1996); 5) aldehydes are often implicated in cardiotoxicity (Carvalho *et al.*, 2004; Luo *et al.*, 2007; Guo and Ren, 2010b). As such, it is of unquestionable worth investigating the role of ABC metabolism in the onset of its toxic effects (**Fig. 1.9**).

Table 1.1. Examples of drugs activated *via* aldehyde metabolites and their associated toxicities

Drugs	Use	Aldehyde metabolite	Toxicity
Cyclophosphamide	Anticancer	Acrolein	Oxidative stress
Ifosfamide	Anticancer	Chloroacetaldehyde	Neuro/nephrotoxicity
Misonidazole	Anticancer	Glyoxal	Polyneuropathy
Sudoxicam	NSAID	Glyoxal	Hepatotoxicity
Felbamate	Epilepsy	Antropaldehyde	Hypersensitivity
Tribromoethanol	Anesthetic	Tribromoacetaldehyde	Hepatotoxicity
Chloral hydrate	Sedative	Tribromoacetaldehyde	Hepatotoxicity
Sorbinil	Aldose redutase inhibitor	Open-chain aldehyde intermediate	Immunotoxicity

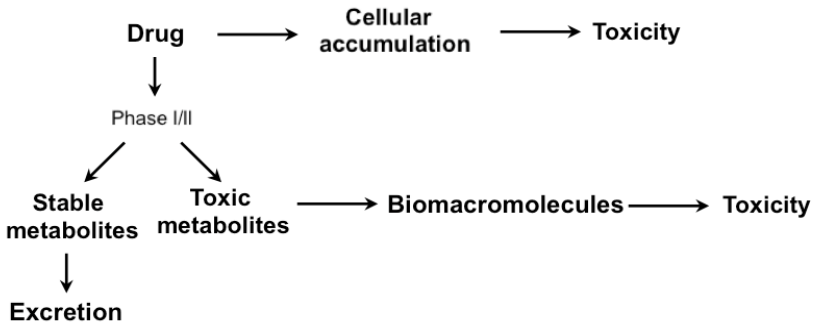

Figure 1.9. Drug bioactivation and toxicity. Toxicity may occur through the parent drug accumulation or by drug bioactivation through formation of reactive (toxic) metabolites, which, if not detoxified can bind covalently to macromolecules.

1.6 Objectives

The fact that ABC-induced HSR and cardiotoxicity mechanisms are relevant and unclear subjects and the necessity for a prospective biomarker of ABC toxicity, especially for preventing chronic events, has established the objectives of the present work. ABC bioactivation to an aldheyde metabolite has never been identified in man, mainly due to the absence of suitable methodological approach. Our initial hypothesis was that this reactive metabolite could be efficiently trapped by *N*-terminal valine of hemoglobin, forming *N*-terminal-valine-abacavir adducts that could be used to quantify ABC bioactivation and plausibly be used as biomarkers of its toxicity. Therefore, the general objective was to give evidence for ABC bioactivation in man, through the identification of ABC adducts. To achieve the main goal it was firstly necessary to develop an analytical methodology to identify ABC adducts, test it and optimize it in an animal model. The identification and quantification of this adducts in HIV-

infected patients represented the second objective (**Fig. 1.10**). Finally, the identification of factors which could influence the formation of ABC adducts were also projected.

Specific aims:

1) Develop a methodology to access ABC adducts
2) Test the methodology and optimize in an animal model
3) Identify ABC adducts in HIV-infected patients
4) Search for factors that may influence the formation of adducts

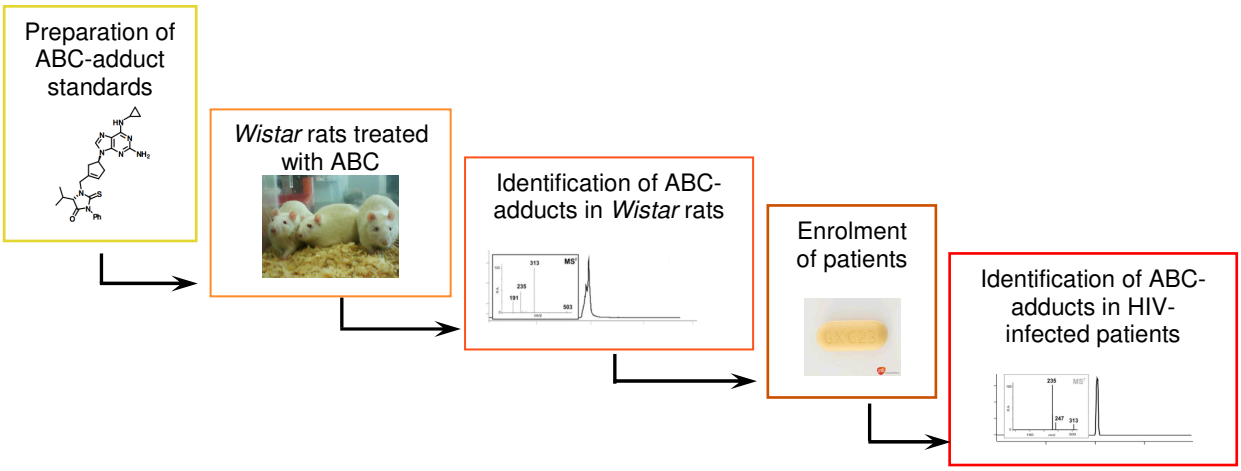

Figure 1.10. Graphic summary of study work plan.

2. Materials and Methods

2.1 Materials

2.1.1 Reagents

All reagents were acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). The solvents were purified and dried when applicable.

Abacavir (ABC) reference was supplied through the program *NIH AIDS Research and Reference Reagent Program* (abacavir, Cat 4680). ABC sulfate was kindly offered by Dr. Frederick Beland (NCTR, Arkansas, USA).

2.1.2 Enzymes

Pronase E (EC 3.4.24.31) and leucine aminopeptidase M (EC 3.4.11.2) have been both acquired from Sigma-Aldrich Co. (St. Louis, MO, USA). Pronase E solution (0.53 mg/mL) was prepared in phosphate buffered saline 10mM (PBS) and leucine aminopeptidase M solution (0.13 mg/mL) was prepared in distilled water.

2.1.3 Consumables

Silica gel plates 60 F254 from *Merck* (Darmstadt, Germany) with a thickness of 0.5 mm were purchased for performing preparative thin layer chromatography (TLC). The plates were observed by ultraviolet light at a wavelength of 254 nm.

Amicon® Ultra-4 Centrifugal Filter Devices, 30,000 MWCO from *Millipore* (Billerica, MA, USA) were used for isolation of albumin from plasma.

C-18 Sep-Pak cartridges from *Waters* (Milford, MA, USA) were used when performing enzymatic hydrolysis, for sample concentration.

2.2 Equipment

2.2.1 High-performance liquid chromatography (HPLC)

The semi-preparative and analytical HPLC analysis of ABC-adducts standards was conducted on an Ultimate 3000 *Dionex* (Sunnyvale, CA, USA) system, consisting of a LPG-3400A quaternary gradient pump, a diode array spectrophotometric detector and an automatic 8125 *Rheodyne* (Rohnert Park, CA, USA) injector. The semi-preparative analyses were performed on a Luna C18 column (250 × 10 mm; 5 µm) and the analytical were in a Luna C18 column (250 mm × 4.6 mm; 5 µm), both from *Phenomenex* (Torrance, CA, USA). Data acquisition and processing was performed on the Chromeleon® 7.1 software from *Dionex* (Sunnyvale, CA, USA).

The quantification of ABC in plasma by HPLC was performed in a *Schimadzu* (Kyoto, Japan) system, consisting of a LC 9-A solvent delivery pump, a 7725i injector, a SPD-6 AV UV-VIS detector and a CTO-10AS VP column oven. Was used a *LiChrospher* 100 RP-18 (250 x 4 mm; 5 µm) column protected by a *LiChrospher* 100 RP-18e (4 x 4mm; 5 µm) guard-column, both from *Merck* (New Jersey, USA.). Data acquisition and processing were performed on the *Shimadzu* Class VP 7.X software. Isocratic elution was with a mixture of methanol:acetonitrile:potassium dihydrogen phosphate 3.7 mM at pH 5.65 (35:10:55; v/v/v), a flow rate of 0.6 mL min at 25 ºC. The UV detector was set at a wavelength of 275 nm.

2.2.2 Liquid Chomatography-Electrospay Ionization-Tandem Mass Spectrometry (LC-ESI-MS)

LC-ESI-MS/MS analyses were performed using a *ProStar* 410 autosampler, two 210-LC pumps, a *ProStar* 335 diode array detector and a 500-MS ion trap mass spectrometer with an ESI ion source from *Varian,* Inc. (Palo Alto, CA, USA). Data acquisition and processing were performed using the *Varian* MS Control 6.9.3 software. The samples were injected *via* an injector with a 20 µL loop from *Rheodyne* (Rohnert Park, CA, USA). A Luna C18 column (150 mm x 2 mm, 3 µm) from *Phenomenex* (Torrance, CA, USA) was used. The MS was operated in the positive ESI mode. The optimized operating parameters were: ion spray voltage, +5.2 kV; capillary voltage, 80 V and RF loading, 70%. Nitrogen was used as the nebulizing and drying gas, at pressures of 45 and 10 psi, respectively. The drying gas temperature was 350 ºC. MS/MS spectra were obtained with an isolation window of 2 Da, excitation energy of 1.7 V and excitation time of 10 ms. The MS^n spectra were obtained with an isolation window of 1.0 Da, excitation energy values of 1.5 and 1.2 V and an excitation time of 10 ms (CID up to MS^3).

2.3 Methods

2.3.1 Preparation of abacavir adduct standards

2.3.1.1 Abacavir generation from abacavir sulphate

ABC sulphate powder (600 mg) was dissolved in water (150 mL), followed by the addition of potassium hydroxide 1M, to reach pH 8. This solution was then extracted with dichloromethane (4 x 200 mL). Subsequently, the organic phase was dried with anhydrous magnesium sulfate and filtered. The solvent was removed under reduced pressure, affording a white solid and the purity of this compound was confirmed by HPLC analysis upon comparison with ABC reference.

2.3.1.2 Swern Oxidation – Preparation of abacavir conjugated aldehyde

Oxalyl chloride (78 µL) was added to anhydrous tetrahydrofuran (THF; 3 mL) in a two-necked flask under nitrogen at -78 ^{o}C, followed by the addition of dimethyl sulfoxide (DMSO; 130 µL). This mixture was kept under stirring for 5 min; a suspension of abacavir (200 mg) in anhydrous THF (9 mL) was

added and stirred at -78 °C, for 1 h. At this stage, anhydrous triethylamine (507 µL) was added to the mixture, the temperature was allowed to rise to 0 °C and stirred for 1 h. Afterwards, distilled water (9 mL) was added and the mixture was extracted with diethyl ether (3 x 100 mL). Anhydrous magnesium sulfate was added to the organic phase to remove water and the solvent was removed under reduced pressure. The mixture was purified by preparative TLC [eluted in dichloromethane/methanol (9:1)], affording a yellow oil. Identification of the obtained compound was confirmed by analytical HPLC analysis upon comparison with a previously prepared standard (Charneira *et al*., 2011).

2.3.1.3 Reaction of ethyl valinate with abacavir-conjugated aldehyde

A solution of ethyl valinate hydrochloride (40 mg) was prepared in phosphate buffer 50 mM at pH 7.4 (800 µL) and subsequently treated with sodium hydrogen carbonate (18 mg) for 30 min at 37 °C. Following the addition of ABC-conjugated aldehyde solution in THF (0.5 mL) the mixture was incubated for 30 min, at 37 °C. Sodium cyanoborohydride (150 mg) was then added and the resulting mixture was re-incubated at this temperature overnight. The solvent was removed under reduced pressure.

2.3.1.4 Abacavir-*Edman* adduct standard

The mixture afforded at **section 2.3.1.3** was dissolved in *N,N*-dimethylformamide (DMF; 1 mL). sodium hydroxide 1M (40 µL) and phenyl isothiocyanate (PTIC; 6 µL) were both added. The solution was subsequently stirred for 2 h at 37 °C and then for 1.5 h at 45 °C. Upon cooling to room temperature, water (2.5 mL) was added and the solution was extracted with ethyl acetate (2 x 2.5 mL). The organic phase was dried under reduced pressure. The ABC-*Edman* adduct was purified by semi-preparative HPLC, using the chromatographic conditions described in **Table 2.1** and its purity was subsequently confirmed upon LC-ESI-MS analysis (**Table 2.2**).

Table 2.1. Chromatographic conditions for HPLC analysis and isolation of ABC-*Edman* adduct standard.

Time (min)	Formic acid 0.1% (%)	Acetonitrile (%)	Flow (mL/min)	UV absorbance (nm)	Temperature (°C)
0	100	0	3	254	25 °C
15	0	100			
17	0	100			
25	100	0			
30	100	0			

Table 2.2. Chromatographic conditions for LC-ESI-MS/MS analysis of ABC-*Edman* adduct standard.

Time (min)	Formic acid 0.1 % (%)	Acetonitrile (%)	Flow (mL /min)	Temperature (°C)
0	100	0	0.2	30
15	0	100		
17	0	100		
25	100	0		

2.3.2 Identification of abacavir-adducts in *Wistar* rats

2.3.2.1 Animal treatment

The animal handling protocol was approved by the Institutional Animal Care and Use Committee.

A group of Wistar rats (10-13 weeks old; three males and three females; 274-388 g) were obtained from the *vivarium* of the Faculty of Medical Sciences, New University of Lisbon, where the animals were kept under controlled temperature with 12/12h light/dark cycles. They received a standard rodent diet and tap water *ad libitum*.

The Wistar rats were exposed to ABC, which was suspended in methyl cellulose (0.2% in water) with 5% of methanol. The rats were administered eight daily intraperitoneal doses of 120 mg ABC/kg body weight. An extra group of each gender (two males and two females) received the vehicle alone. Two hours after the last treatment, the rats were anesthetized (60 mg/kg of pentobarbital), the chest cavity was opened and blood was collected by cardiac puncture into EDTA tubes. The blood samples were centrifuged at 3,000 *g* for 10 min, to separate plasma from blood cells. The samples were aliquoted and stored at -80 °C until Hb isolation[1].

2.3.2.2 Isolation of hemoglobin (Tornqvist *et al.*, 2002)

Aliquots of the red blood cells (400 µL) were washed with sodium chloride 0.9% (3 x 400 µL). The saline solution was discarded and Milli-Q water (600 µL) was added to each sample, to promote cell lysis. Next, a hydrochloric acid 50 mM in 2-propanol solution (6mL) was added to 1 mL of the lysate and the mixture was centrifuged at 3000 *g* for 10 min, to remove cell membranes. To the supernatant was added iced ethyl acetate (40 mL) to precipitate Hb and the pellet was washed with 20 mL of *n*-pentane and finally dried under reduced pressure.

[1] The blood samples were already available at the Pharmacology lab.

2.3.2.3 Serum albumin isolation (Lindstrom *et al.*, 1998)

To the rat plasma (400 µL) was added a saturated ammonium sulfate solution (400 µL). The solution was centrifuged at 900 *g*, 4 °C, for 30 min to remove the immunoglobulins. The supernatant was filtered through an *Amicon*® centrifugal filter with centrifugation at 3800 *g*, 4 °C, for 20 min and then the resulted solution was dried under reduced pressure.

2.3.2.4 Detachment of *N*-terminal valine adducts from hemoglobin - *N*-Alkyl *Edman* degradation (Charneira *et al.*, 2012)

Each Hb sample (50 mg) from **section 2.3.2.2** was dissolved in DMF (1.5 mL), followed the addition of sodium hydroxide 1M (65 µL) and PTIC (10 µL). The samples were subsequently stirred for 2 h at 37 °C and for 1.5 h at 45 °C. Upon cooling to room temperature, water (2 mL) was added and the resulted mixtures were extracted with ethyl acetate (2 x 1 mL). The organic phases were dried under reduced pressure, resuspended in methanol (50 µL) and were analyzed by LC-ESI-MS/MS using the chromatographic conditions described in **Table 2.2**.

2.3.2.5 Hydrolysis of serum albumin to amino acids (Tsao and Otter, 1999)

Serum albumin samples (10 mg) from **section 2.3.2.3** were dissolved in PBS 10mM (3.5 mL) and solutions of Pronase E (190 µL, 0.53 mg/mL) and leucine aminopeptidase M (80 µL, 0.13 mg/mL) were added. The solution was stirred at 37 °C overnight. The enzymatic hydrolysate was concentrated in a C-18 Sep-Pak cartridge. The cartridge was conditioned with methanol (3 mL), followed by water (6 mL). The sample was then loaded, and the cartridge was rinsed with water (1 mL) and methanol (2 mL). The methanolic eluate was dried under reduced pressure, reconstituted in 60 µL acetonitrile/0.1% aqueous formic acid (1:1) and analyzed by LC- ESI-MS/MS through the chromatographic conditions described in **Table 2.3.**

Table 2.3. Chromatographic conditions used for LC-ESI-MS/MS analysis of rat serum albumin hydrolisates.

Time (min)	Formic acid 0.1 % (%)	Acetonitrile (%)	Flow (mL /min)	Temperature (°C)
0	95	5	0.2	30
5	95	5		
35	30	70		
37	0	100		
45	0	100		

2.3.2.6 Quantification of abacavir concentration in *Wistar* rats

2.3.2.6.1 Preparation of stock and calibration solutions.

Stock solutions of ABC (1 mg/mL) were prepared in methanol and were stored at -80° C until handling.

Different calibration solutions, to obtain a calibration curve covering the range from 0.005 to 20 mg/L, were prepared by dilution of the ABC solution in plasma from rats not exposed to ABC.

All calibration solutions were heated for 60 minutes at 60 ºC before being submitted to the extraction procedure.

2.3.2.6.2 Samples extraction procedure and HPLC analyses.

A liquid-liquid extraction procedure was performed. Briefly, dichloromethane was added to human plasma (500 µL) in a proportion of 1:10, in a 10 mL glass tube. The solution was mixed on a vortex mixer and centrifuged at 2 000 *g* for 5 min, at 4ºC. The organic phase was recovered, and the aqueous phase was re-extracted. The two organic phases were combined. The solvent was dried under reduced pressure, reconstituted in potassium dihydrogen phosphate 3.7 mM/methanol (1:1; 150 µL) and 100 µL where injected in the HPLC.

2.3.2.7 Identification and quantification of abacavir-adducts in HIV-infected patients

2.3.2.7.1 Enrollment of patients and, demographic and clinical data collection.

The protocol received prior approval from the Ethics Committee of *Centro Hospitalar de Lisboa Central, EPE*. The patients gave their written informed consent and adherence was controlled through a questionnaire. All eligible patients (5 men and 5 women) were adults with documented HIV-infection who had received continuous treatment with ABC-containing cART regimens (300 mg twice daily or 600 mg once daily) for more than 1 month, regardless of past therapeutic history. Exclusion criteria were being under 18 years of age, having AIDS-defining conditions or compliance issues. A control group (2 healthy volunteers) was also included in the study. From all the patients relevant data were collected: age, sex, weight, height, ethnicity, alcohol consumption, injection drug use, smoking habits, opportunistic infections, co-morbidities, adverse events, antiretroviral medication, and other analysis consider relevant for the study (see Annex I – Case Report Form).

Blood samples (2 mL) were collected in EDTA tubes. The samples were centrifuged at 3 000 *g* for 10 min, 4 °C. The samples of plasma and cells were aliquoted and store at -80 °C until use.

All patients' samples were heated for 60 min, at 60ºC, for HIV inactivation, just before handling at room temperature.

2.3.2.7.2 Abacavir-*Edman* adduct analysis in HIV-infected patients

Identification and quantification of the ABC-*Edman* adduct was conducted in three steps: a. hemoglobin isolation and purification from blood samples (see section **2.3.2.2)**; b. detachment of *N*-terminal valine adducts from haemoglobin (see **section 2.3.2.4)**; and c. analysis of the detached adducts by LC-ESI-MS/MS (see **section 2.3.2.4).**

For ABC-*Edman* adducts quantification in patients a stock solution of ABC-adduct standard (1 mg/mL) was prepared in methanol. Solutions were stored at -20 ^{o}C until analyze. Afterwards, different calibration solutions, to perform a calibration curve covering the range from 0.25 to 2.5 ng/L, were prepared by dilution from the ABC-adduct standard solutions in methanol.

2.3.2.7.3 Quantification of abacavir plasma concentration in HIV-infected patients

The quantification of ABC in plasma from HIV-patients was performed in three steps: **a)** preparation of stock and calibration solutions; **b)** Samples extraction and **c)** HPLC analysis (see **section 2.3.2.6**).

3. Results

3.1 Preparation of abacavir adducts standards

ABC-conjugated aldehyde was prepared upon ABC Swern oxidation, using trietilamine, as base. The resulting mixture from Swern reaction was purified by preparative TLC, which allowed the isolation of the ABC-conjugated aldehyde with 18% yield. For the preparation of the stabilized adduct (**Fig. 3.1**), ABC-conjugated aldehyde in THF was initially incubated with a solution of ethyl valinate in a phosphate buffer 50 mM and then subjected to reduction with sodium cyanoborohydride to stabilize the Shciff base formed. The reaction mixture was then submitted to *N*-Alkyl *Edman* degradation, using the phenyl isothiocyanate as the derivatizing agent (Chevolleau *et al.*, 2007), and following purification by semi-preparative HPLC the ABC-*Edman* adduct was obtained.

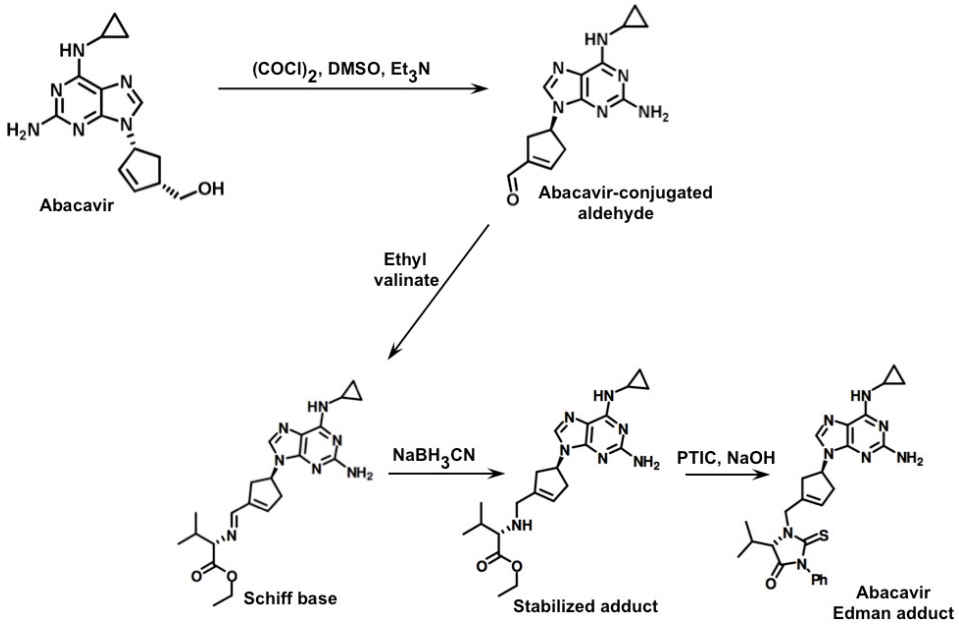

Figure 3.1. Formation of abacavir-*Edman* adduct. Swern oxidation of abacavir into its conjugated aldehyde derivative followed by formation of the Schiff base upon reaction with ethyl valinate, which is then stabilized by reduction and derivatizated with phenyl isothiocyanate in a basic medium.

The purified compound was then analyzed by LC-ESI-MS/MS. The ionic chromatogram exhibited only one signal at 9.7 min, confirming the purity of the obtained product, the ABC-*Edman* adduct standard. The identification of this adduct was based upon undistinguishable mass spectra and identical retention time, when compared with previously prepared synthetic standard (**Fig. 3.2**). The mass spectra exhibited a protonated molecule at *m/z* 503 that upon tandem mass fragmentation afforded three fragment ions (**Fig. 3.3**), stemming from loss of the purine moiety (*m/z* 313), loss of the ABC moiety (*m/z* 235), and cleavage of the purine-cyclopentene bond, with protonation on the purine

moiety (*m/z* 191), respectively. Taken together, all data are in accordance the ones described by Charneira *et al*. (2011), confirming that the isolated product is the ABC-*Edman* adduct (Charneira *et al*., 2011).

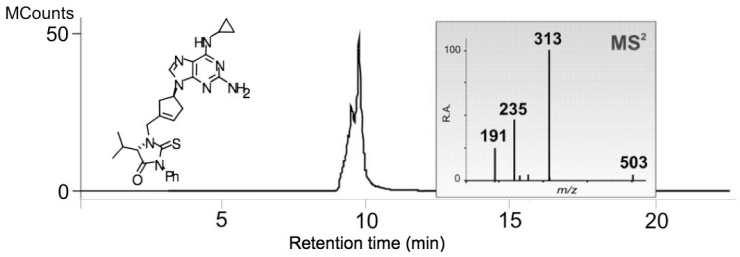

Figure 3.2. LC-ESI-MS/MS chromatogram of ion *m/z* 503 from ABC-*Edman* adduct standard.

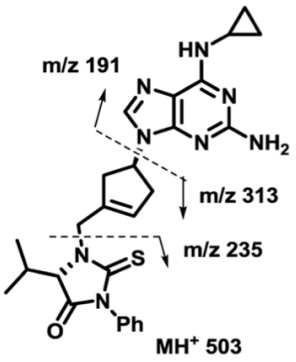

Figure 3.3. LC-ESI-MS/MS fragmentation pattern for the ABC-*Edman* adduct (protonated molecule *m/z 503*).

3.2 Identification of abacavir-adducts in *Wistar* rats

3.2.1 Identification of abacavir-*Edman* adducts with *N*-terminal valine of hemoglobin

To obtain evidence for ABC-conjugated aldehyde ability to modify proteins *in vivo*, three male and three female Wistar rats were treated with eight daily doses of ABC (120 mg/kg body weight) and the formation of covalent adducts with the *N*-terminal valine of Hb was investigated by LC-ESI-MS/MS. The LC-ESI-MS/MS analysis of the ion at m/z 503 (corresponding to the protonated molecule of the expected adduct) allowed the identification of the *N*-terminal valine adduct from two (one male and one female) out of the six rats. Based on identical retention times (9.7 min) and mass spectra (**Fig. 3.4**) when compared with the synthetic standard prepared in **section 2.3.1**, was possible the unequivocal identification of ABC-adducts in *Wistar* rats. The corresponding MS/MS spectra of ion m/z

503 consistently presented three fragment ions, stemming from loss of the cyclopropylaminopurine moiety (m/z 313), loss of the abacavir moiety (m/z 235), and cleavage of the purine-cyclopentene bond, with protonation on the purine moiety (m/z 191). These signals were absent from all the control rat samples.

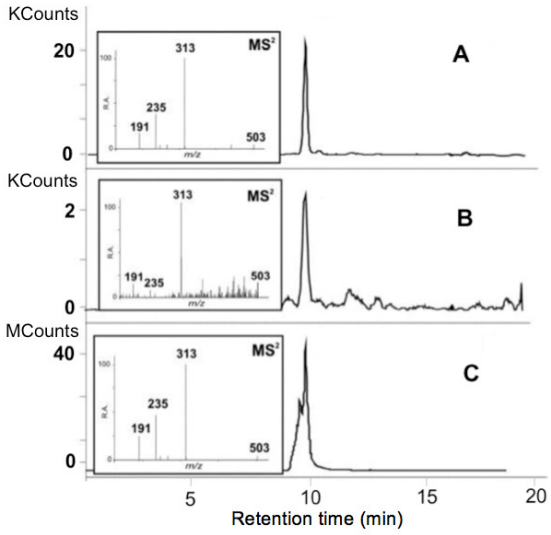

Figure 3.4 LC-ESI-MS/MS analysis from hemoglobin of the Wistar rats exposed to abacavir. A. female Wistar rat; **B**. male Wistar rat and, **C**. abacavir-*Edman* adduct standard.

3.2.2 Identification of abacavir adducts with other amino acids from serum albumin

To search for other potential targets of ABC-conjugated aldehydes, the isolated rat serum albumin was hydrolyzed to free amino acids by an adaptation of reported methodologies, using a combination of pronase E and leucine aminopeptidase M, to ensure the endo- and exopeptidase activities required for complete hydrolysis (Tsao and Otter, 1999). Following concentration of the enzymatic hydrolysate in a C-18 Sep-Pak cartridge, the methanolic eluate was analyzed by LC-ESI-MS/MS to search for the presence of plausible adducts with amino acids containing nucleophilic side chains (**Fig. 3.5**). However no adducts were detected in the rat albumin hydrolysate.

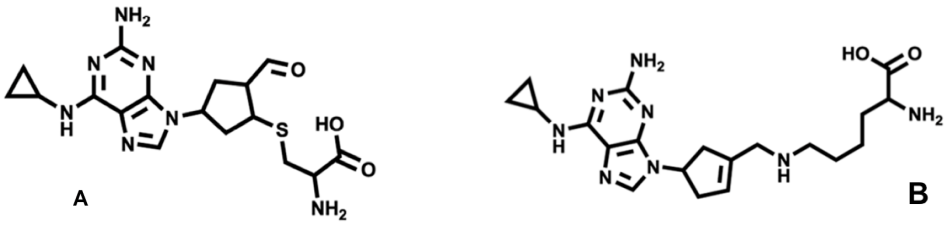

Figure 3.5 Structures of the covalent adducts expected upon A) Michael addition of cysteine to the double bound of abacavir-conjugated aldehyde, **B**) Shiff base formation, upon lysine addition to the abacavir carbonyl group, followed by reductive stabilization.

3.2.3 Quantification of abacavir in plasma from *Wistar* rats

Thought analytical HPLC analysis was possible the quantification of ABC concentration in plasma from the Wistar rats treated with ABC. Based on identical retention times (8.3 min) when compared with the standards solutions was possible the identification of ABC peak, and by a calibration curve ABC plasma concentrations were estimated to range between 7.5 and 14.4 mg/L. For rats with ABC-*Edman* adducts the mean ± standard deviation (SD) of ABC concentration was 10.8 ± 1.73 mg/L. In those rats which were not identified with adducts, the mean ± SD of ABC concentration was 13.5 ± 5.7 mg/L. No differences were found (unpaired T-test) between ABC concentration in rats with ABC-*Edman* adducts and those in which ABC-Edman adducts were not identified.

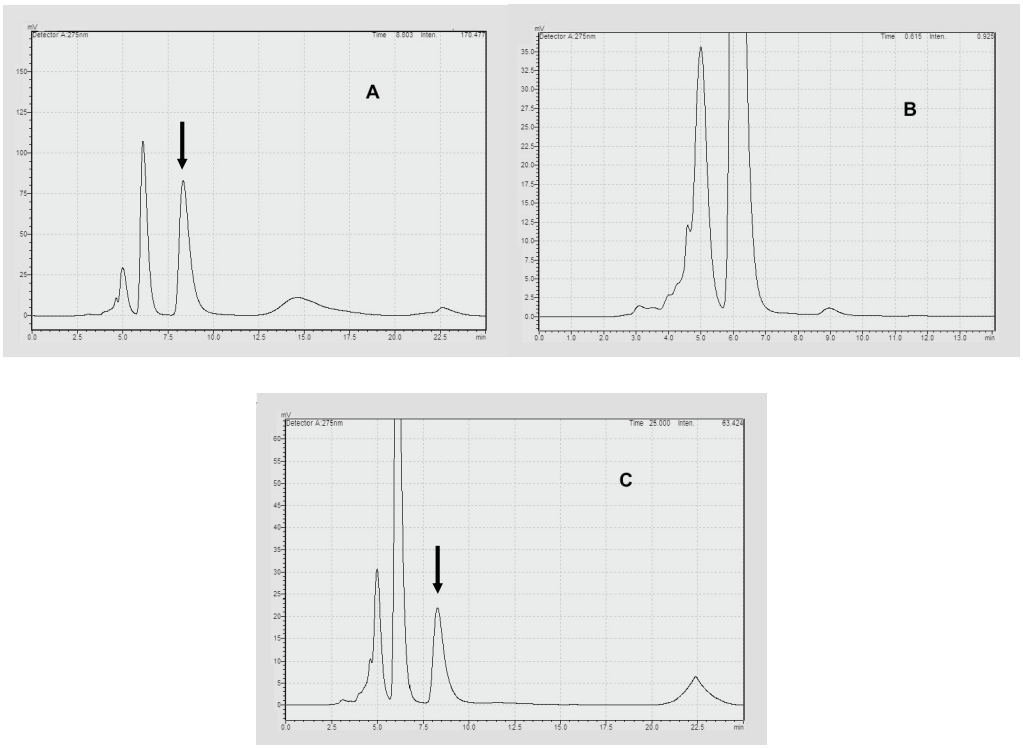

Figure 3.6. Quantification of abacavir concentration in Wistar rats exposed to abacavir. A, Chromatogram obtained following analytical HPLC analysis of a rat exposed to abacavir. The peak identified with an arrow corresponds to abacavir; **B**, Chromatogram from a rat not exposed to abacavir; **C**, Chromatogram from a calibration solution in a concentration of 1.5 mg/L (retention time 8.3 min).

3.1 Identification and quantification of abacavir-adducts in HIV-infected patients

3.1.1 Patients demographic and clinical data

The demographic and clinical data collected from patients who participate in the study are gathered in **Table 3.1**. The patients were 49.7 ± 16.5 years old, half patients were female, and one patient was black. Six patients developed adverse events, which were not related with ABC. Six patients developed opportunistic infections (acute bronchitis, tuberculosis, pneumonia, candidiasis, syphilis and herpes) and 8 developed associated pathologies (major depression, hepatitis C, hypertension, diabetes, kaposi sarcoma, dyslipidemia, and hepatomegaly). Though the clinical analysis of function liver enzymes it was possible to infer that all the patients had a normal liver function. Three patients presented CD4 count lower (< 350 cell.mm^3) that the recommended (DGS, 2012). Mostly liver function tests where in their normal range.

Table 3.1. Demographic and clinical data from HIV–infected patients on abacavir included in the study.

[1] F, female; M, male.
[2] C, caucasian; B, black.
[3] OD, once a day (600 mg); BID, twice a day (300 mg).
[4] cART, combined antiretroviral therapy; 1, lamivudine boosted with a protease inhibitor; 2, lamivudine and tenofovir boosted with a protease inhibitor; 3, lamivudine and efavirenz and, 4, lamivudine, tenofovir and efavirenz.
[5] Y, yes; N, no.
[6] α, levels are in the normal range; β, levels are out of the normal range.
AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; γ-GT, gamma-glutamyl transferase; ALP, alkaline phosphatase; LDH, Lactate dehydrogenase.

Patient	Age (years)	Sex[1]	Ethnicity[2]	ABC Schedule[3]	cART[4]	Adverse events[5]	Opportunistic infections[5]	Associated pathologies [5]	Alcohol consumption[5]	Smoking habit[5]	Abused drugs[5]	CD4 count (cell.mm^3)[6]	AST (U/L)[6]	ALT (U/L)[6]	γ-GT (U/L)[6]	ALP (U/L)[6]	LDH (U/L)[6]	Total bilirubin (mg/dL)[6]
A	41	M	C	OD	1	N	Y	Y	N	Y	Y	α	α	α	α	α	α	β
B	42	F	C	OD	1	N	Y	Y	N	Y	Y	α	α	α	α	β	-	α
C	56	M	C	OD	2	Y	N	Y	Y	Y	N	β	α	α	α	α	α	β
D	38	M	C	BID	3	Y	Y	Y	N	Y	N	α	α	α	α	α	-	α
E	58	F	C	BID	1	Y	Y	Y	Y	N	N	β	α	α	β	β	α	β
F	34	F	B	OD	2	N	Y	N	N	N	N	α	α	α	α	α	α	α
G	70	F	C	OD	2	Y	Y	Y	N	N	N	β	α	α	α	α	α	-
H	38	F	C	OD	4	N	N	Y	N	N	N	α	α	α	α	β	α	α
I	37	M	C	OD	3	Y	N	N	Y	N	Y	α	α	α	α	α	α	α
J	83	M	C	OD	2	Y	N	Y	Y	N	Y	α	α	α	α	α	α	β

3.1.2 Abacavir-adduct analysis in HIV-infected patients

To obtain evidence for ABC bioactivation to a reactive aldehyde metabolite in man, the presence of ABC-*Edman* adducts with the *N*-terminal valine of Hb was investigated in ten HIV-infected patients (5 men and 5 women; **Table 3.1**).

Through *N*-alkyl *Edman* procedure, for specific detachment of adducts from Hb, and LC-ESI-MS/MS analysis was possible to identify, for the first time, ABC-*Edman* adducts in three patients (two men and one woman; **Table 3.1, patients A, B and C).** LC-ESI-MS/MS analysis of the MS^3 transition of the protonated molecule [*m/z* 503 → 313 → 235] allowed the unequivocal identification of this adduct based upon undistinguishable mass spectra and identical retention time, when compared with the previously prepared synthetic standard in **section 2.3.2**. Specifically, under the chromatographic conditions used, all three positive samples (**patients A, B and C**) and the synthetic standard displayed a signal at 10.5 min, whose MS^3 spectrum consistently presented a characteristic fragments ion at *m*/*z* 235 and 313 (**Fig. 3.5**). This signal was absent from the control human samples (**Fig. 3.5 B**).

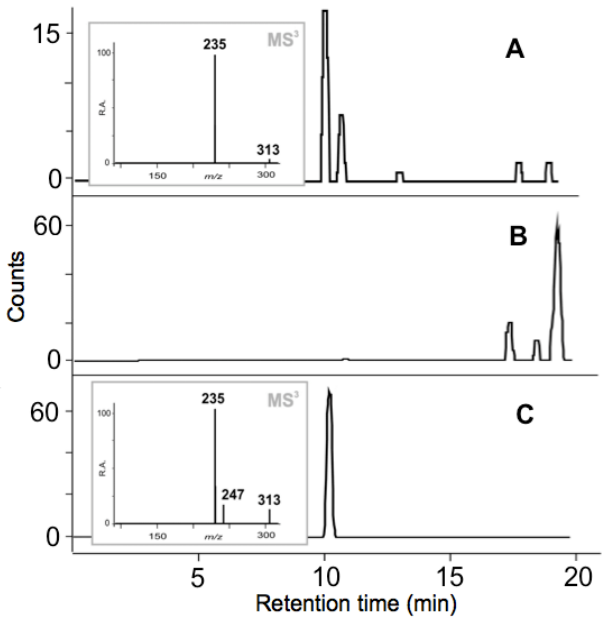

Figure 3.7. Ionic chromatogram obtained following LC-ESI-MS/MS analysis of the MS^3 transition for the protonated molecule of the abacavir-valine *Edman* adduct. A. patient on abacavir; **B.** healthy volunteer not exposed to abacavir, and **C.** abacavir-*Edman* adduct standard.

Upon calibration curve procedure it was possible to quantify the concentration of ABC-adducts in HIV-patients. The three patients (two man and one woman) where were identified the ABC-adducts with Hb reach concentrations of 7 fmol/mg (patient **A**), 2.5 fmol/mg (patient **B**) and 3.3 fmol/mg of Hb (patient **C**).

3.1.3 Quantification of abacavir in plasma of HIV-infected patients

Through analytical HPLC analysis it was possible the quantification of ABC concentration in plasma from all the ten patients (**Fig. 3.5)** included in the study. The ABC plasma concentrations ranged between 0.04 and 1.8 mg/L. For patients with ABC-*Edman* adducts the mean ± SD of ABC concentration was 0.2 ± 0.08 mg/L. For patients who were not identified with adducts, the mean ± SD of ABC concentration was 0.3 ± 0.2 mg/L. No significant difference were found between the concentrations of ABC in patients with ABC-*Edman* adducts and those without adducts.

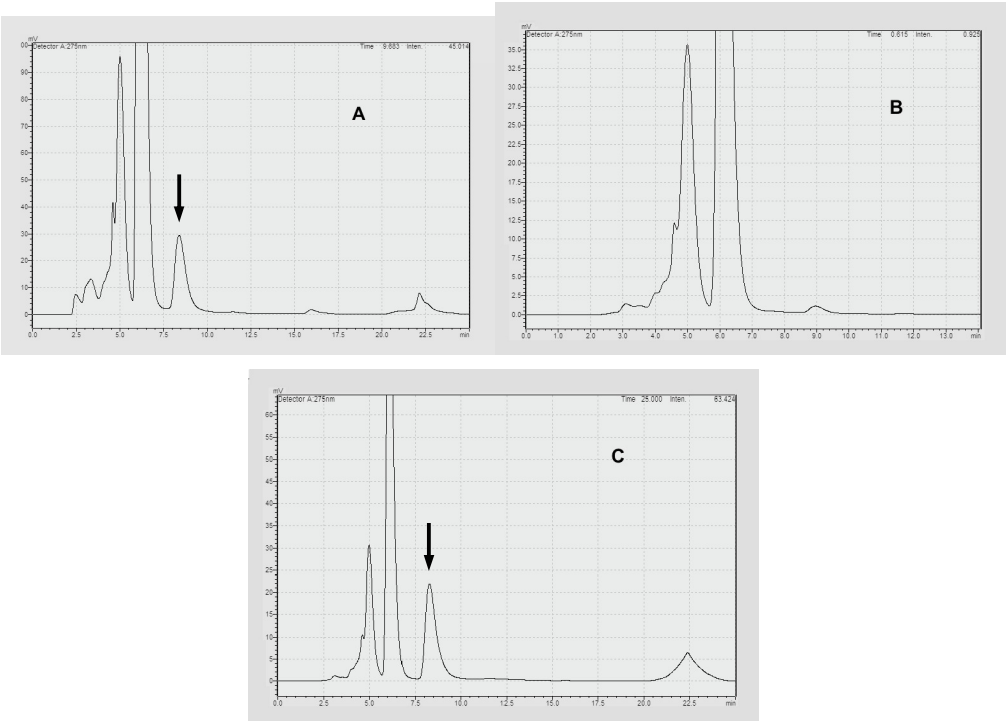

Figure 3.8. Quantification of abacavir concentration in HIV-infected patients. A, Chromatogram obtained following analytical HPLC analysis of a HIV-infected patient on abacavir. The peak identified with an arrow corresponds to abacavir; **B,** Chromatogram from plasma of a healthy volunteer and **C,** Chromatogram from a calibration solution in a concentration of 1.5 mg/L (retention time 8.3 min).

4. Discussion

The exact mechanism underlying ABC toxic events is not clear yet. Nevertheless, it has been suggested that a reactive metabolite produced via ABC-bioactivation might have a key role at the onset of these adverse effects. A decade ago, Walsh *et al*. (2002) have proposed that ABC-bioactivation could take place through ADH mediation. However, the confirmation of this hypothesis only recently emerged, when Charneira *et al.* (2011) unrevealed the mechanism of an ABC reactive metabolite formation – the conjugated aldehyde. ABC-conjugated aldehyde is a short-lived specie and therefore not detectable in man. However, it binds covalently to biological macromolecules (Charneira *et al*., 2011); affording protein adducts which can be quantified in fluids and tissues. So far, there are no similar studies in man reported in literature, probably due to the need of cooperation between Chemists, who synthesize the bioanalytical standards for assessing bioactivation and electrophile metabolites, Pharmacologists and Clinicians who aim at translating basic molecular discoveries in valuable tools for clinical practice application.

Initial Strategy

The drug-protein adducts are broadly studied in blood proteins, mainly albumin and Hb. Both proteins are abundant in blood and have a relatively long life span. Based on *in vitro* studies (Charneira *et al*, 2011) the ABC-conjugated aldehyde was anticipated to react with nuchleophilic amino sites of proteins (*N*-terminal valine of Hb and lysine side chain) via Schiff base formation and with sulfhydryl groups of cysteine *via* Michael addition to the conjugated double bound. These residues are primary sites of reaction with electrophiles and a good model to monitor bioactivation. (Davies *et al*., 2009).

Hb is often used as an easily accessible model for indirect biomonitoring of reactive metabolites, particularly by investigating covalent modification at the *N*-terminal valine residues (Davies *et al*., 2009). Given that the pKa values of the α-amino groups from the *N*-terminal valine residues in proteins (ca. 7.80) are close to that of the blood pH (Tornqvist *et al*., 2002; Davies *et al*., 2009), a large fraction of these residues are unionized and thus have nucleophilic character. For these reasons and for the availability of a mild, simple, and sensitive post-modification procedure capable of selectively detaching valine adducts from the protein (*N*-Alkyl *Edman* degradation), Hb adducts are extensively used as biomarkers of exposure to toxicants (Tornqvist *et al*., 2002; Boysen *et al*., 2007; Chevolleau *et al*., 2007).

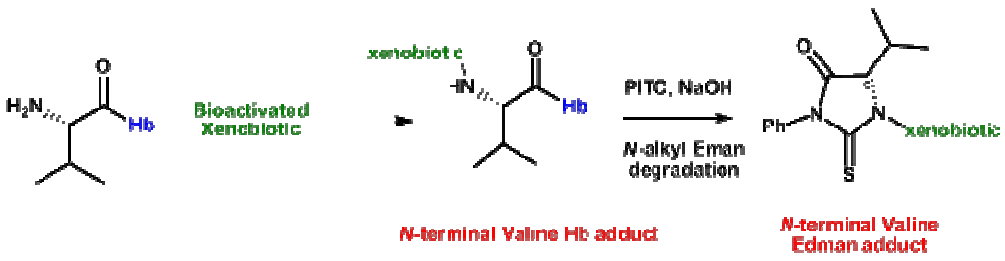

Figure 4.1 Xenobiotic-derived *Edman* adducts. Schematic representation of the formation of *N*-terminal valine hemoglobin (Hb) adducts with bioactivated xenobiotics and their detachment from the protein upon N-alkyl *Edman* degradation. PTIC, phenylisothiocyanate.

The detection of *N*-terminal valine *Edman* adducts can be achieved by LC-ESI-MS analysis upon comparison with a synthetic satandard. Therefore, towards the identification of this adduct in biological samples a prior step, involving the synthesis of a standard of ABC-conjugated aldehyde with valine, was needed for this purpose.

Preparation of ABC-*Edman* adduct standard

The *Swern* reaction is frequently used as a fundamental procedure to the oxidation of alcohols to aldehydes (Crich and Neelamkavil, 2001). Therefore, this reaction was used to perform the oxidation of ABC to its conjugated aldehyde (Charneira *et al*., 2011). The ABC-conjugated aldehyde was then incubated with ethyl valinate affording a Sciff base (**Fig. 3.1**), which is prone to hydrolysis. Therefore a subsequent step of reduction, using sodium cyanoborohydride, is needed to afford a stabilized adduct which was then subjected to *N*-Alkyl *Edman* degradation, allowing the isolation of ABC-*Edman* adduct by semi-preparative HPLC.

Identification of ABC-*Edman* adduct in *Wistar* rats

Thereafter, it was necessary optimize the analytical conditions to detect this adducts in HIV-infected patients. Towards this goal we decided to use blood samples from *Wistar* rats were treated with ABC. This decision was based on the fact that these animals could be exposed to a dose of ABC ca.10-fold higher than the expected systemic exposure in humans administered with ABC, in order to maximize the likelihood of obtaining a detectable response in a short time span, as is expected the metabolism in rat is 10 times higher than in man.

The *Wistar* rats (three female and three male) were treated with eight daily doses of ABC (120 mg/kg body weight), their Hb was isolated, subjected to *N*-Alkyl *Edman* degradation and the presence of ABC-*Edman* adducts was screened by LC-ESI-MS/MS.

The LC-ESI-MS/MS analysis of Hb by comparison with the synthetic standard prepared, allowed the identification of ABC-*Edman* adducts in one female and one male rat, based on identical retention times (9.7 min) and MS/MS spectra. The spectra consistently presented three fragment ions - m/z 313, m/z 235, and m/z 191 (Fig. 3.4) - and unequivocal identification of ABC-*Edman* adducts was possible.

These findings represented the first *in vivo* evidence of both the involvement of the conjugated aldehyde intermediate in the metabolic activation of ABC and of its ability to modify covalently the *N*-terminal valine residues of Hb.

Identification of ABC-albumin adducts in *Wistar* rats

ABC-conjugated aldehyde has also been proved of capable of reacting with N-acetylcysteine *in vitro,* through Michael addition to the conjugated aldehyde (Charneira *et al*., 2011). This observation was consistent with the known propensity of conjugated carbonyls to undergo 1,4-addition by soft

nucleophiles, which explains why sulfhydryl groups are considered toxicologically relevant targets for α,β-unsaturated aldehydes (LoPachin *et al.*, 2008). Since albumin residues contain a free, solvent-accessible, sulfhydryl group (Cys-34) (Dooley *et al.*, 2007), they are easily subjected to nucleophilic attack. To explore this issue, the rat albumin was hydrolyzed enzymatically to amino acids and analyzed by LC-ESI-MS/MS in search for potential products of i) Michael addition of cysteine to the conjugated double bond of aldehyde (**Fig 3.5A**) and ii) lysine addition to the carbonyl group of aldehyde with Schiff base formation (**Fig 3.5B**).

To search for ABC-cysteine adducts in plasma from the ABC-treated rats, the LC-ESI-MS/MS methodology was similar to the one used for the successful detection and characterization of albumin-cysteine and lysine adducts formed *in vitro* for another antiretroviral, the NNRTI Nevirapina (NVP) (Antunes *et al.*, 2010). Despite the previous evidence that the conjugated aldehyde reacts with cysteine and lysine (Charneira *et al.*, 2011) *in vitro*, none of these adducts were identified in rat albumin hydrolysates. A possible explanation for this negative result in the identification of ABC-lysine/cysteine adducts suggests can be the lower sensitivity of the analytical method for these adducts when compared with the one obtained for analyzing *N*-terminal valine adducts. Indeed, a recognized advantage of analyzing *N*-terminal valine adducts in hemoglobin is the simplicity of sample treatment, combined with the selective extraction of these adducts to an organic solvent, which minimizes matrix interferences and allows significant adduct enrichment, thereby enabling high levels of sensitivity in MS-based analytical methods. Indeed, regardless of the potential toxicological relevance of ABC-cysteine/lysine adducts, the efficient monitoring of such adducts *in vivo* appears unlikely at the present stage. Therefore, the analysis of ABC-*Edman* adducts with *N*-terminal valine of Hb was elected for searching for evidence for ABC-conjugated aldehyde formation in HIV-infected patients.

Determination of ABC blood concentration in *Wistar* rats

In order to understand if the concentration of ABC influences adduct formation, a method for the quantification of ABC concentration was developed and tested on the blood of *Wistar* rats. The methodology for the quantification of ABC concentration in plasma was based in the one described by Sudha *et al.* (2010), but in order to achieve the best sensitivity and selectivity, several parameters were modified. For the sample extraction procedure several approaches were screened: extraction after precipitation of plasma proteins by methanol and acetonitrile, liquid-liquid extraction with dichloromethane and solid-phase extraction. The best results were achieved with the liquid-liquid extraction with dichloromethane. The chromatographic conditions were also tested. Several mobile phases were tested and in different percentages, methanol:water, acetonitrile:water, methanol: phosphate buffer 3.7 mM and methanol:acetonitrile:phosphate buffer 3.7 mM, which was the one with the best results. The pump flow and the column temperature were also optimized. Nevertheless, was not found any relation between ABC concentration and ABC-*Edman* adducts formation. This result does not mean that there is no relation, once it is necessary to consider, that the number of samples (n = 6) was small, which difficult an analysis statistically significant.

Identification of ABC-*Edman* adduct in HIV-infected patients

The ABC-*Edman* adduct was then investigated in HIV-infected patients, through the optimized procedure in rat 1) the isolation of Hb, 2) *N*-Alkyl *Edman* degradation and 3) LC-ESI-MS analysis of ion m/z 503 through comparison with the ABC-*Edman* adduct standard. However, using these analytical conditions the identification of adducts was very difficult due to matrix interferences. As such we had to improve the method selectivity by following the MS^3 transition of the protonated molecule [*m/z* 503 → 313 → 235]. Using this improved methodology, the ABC-*Edman* adduct was unambiguously detected in three out of ten patients. These results represent the first report on the involvement of a conjugated aldehyde in the metabolic activation of ABC in man. The fact that, only 30% of the patients had detectable adduct levels implies, as expected, a high degree of inter-individual variability in ABC activation/detoxification.

Drug pharmacokinetics (PK) and consequently drug response can be influenced by several factors as age, sex, race, drug interactions or pathological states. In fact, those patients had several different associated co-pathologies such as hepatitis C virus-infection, hypertension, diabetes or depression. It is also important to consider that as immunocompromised persons, HIV-infected patients have a high risk of developing several opportunistic infections, tuberculosis or pneumonia. These pathological status can by themselves introduce variability in drug pharmacokinetics. Moreover the therapies used to treat opportunistic infections can lead to drug interactions. Some patients also had habits like smoking, alcohol consumption or drug abuse. All these factors can alter the patient's pharmacokinetics and/or their tolerance to drug-induced toxicity. Moreover, the enzymes responsible for ABC metabolism (e.g. ADH) are polymorphic, which also can influence drug PK (Rao *et al.*, 2007). Also, HIV infection increases risk of drug toxicity (Koopmans *et al.*, 1995). For instance, glutathione (GSH) deficiency has been suggested to be a main factor on the increased frequency of this reactions (Koopmans *et al.*, 1995). This antioxidant plays an important role in the detoxification of a variety of electrophilic compounds via catalysis by glutathione-S-transferases and glutathione peroxidases.

Indeed, further work is required to clarify the implications of this interpatient variability for toxicity. Other interesting fact is that the three patients that have the ABC-*Edman* adduct were on an ABC 600 mg OD schedule. Clearly, an OD regimen has the advantage of therapeutic adherence and a lower risk for the development of resistance, but it also raises the risk related to toxicity, especially for drugs with a narrow therapeutic window and higher variability degree in its plasma concentrations, as is the case of ABC. The 600 mg OD originates, for example, a higher maximum plasma concentration than the BID regimen, which can influence the metabolism and consequently toxicity.

ABC concentration was also quantified in HIV-infected patients, but as in rats no relation between patients with adducts and patients without those was found.

Is well established that bioactivation of drugs is related to adverse drug events and therefore it is important to consider this hypothesis for ABC. Several evidence support this hypothesis as: 1) the

exposure to aldehydes, either xenobiotic molecules or endogenous intermediates, has been associated with induced allergenic responses (O'Brien *et al.*, 2005) and cardiovascular pathologies (Luo *et al.*, 2007; Guo and Ren, 2010a); 2) aldehydes have been implicated as reactive and capable of covalent binding to proteins and this, in turn, has been suggested to underlying clinical adverse events induced by several drugs (O'Brien *et al.*, 2005). As the case of ethanol, in which acetaldehyde-protein adducts formation has been proposed as a factor for the immune-mediated hepatitis observed with chronic alcohol consumption (Yokoyama *et al.*, 1993); 3) the immunogenic effects of ABC are dose-dependent (Norcross *et al.*, 2012b; Ostrov *et al.*, 2012), and 4) not all HLA-B*57:01 carriers develop HSR to ABC (Nolan, 2009), thus different mechanisms can be considered.

The most important concern of ABC use is HSR. Several hypotheses have been proposed for it underlying mechanism.(Pirmohamed *et al.*, 2002; Pichler *et al.*, 2006; Uetrecht, 2008). However, none of them provided a convincing explanation of how ABC induces adverse reactions through the activation of CD8+ cells in a strictly HLA-B*57:01–restricted manner (Chessman *et al.*, 2008). For the hapten hypothesis to be applyed, it would be necessary an HLA-specific drug haptenated peptide, which until this date had not yet been identified. However, the ABC-conjugated aldehyde metabolite indirectly identified in human samples as ABC-*Edman* adduct has that ability, Adam *et al.* (2012), thought an *in vitro* study, has refuted the hapten hypothesis for ABC-induced HSR. As the isoform of ADH that is involved in ABC metabolism it still unknown, he tested the expression of six different ADH isoenzymes (ADH1A, ADH1B, ADH1C, ADH4, ADH5 and ADH6) in various subsets of immune cells. Only ADH5 was expressed. They have also shown that even using ADH or proteasome inhibitors, a stimulation of ABC-reacting T-cell still exists (Adam *et al.*, 2012). The reactivity of ABC-specific T-cell was affected by the inhibition of TAP and the absence of tapasin, suggesting that the ABC presentation depends on the MHC class I pathway. These findings had already been described by others (Chessment et al, 2008). Nevertheless, the inhibition of TAP and tapasin not only affected the peptide loading, but also reduced the expression of HLA molecules on the cell surface. It is possible that the effect of TAP and tapasin inhibition is linked to the density of HLA molecules expressed on the cell surface rather than to peptide processing and uptake into the endoplasmatic reticulum. Also, for the PI model to be applyed, ABC would need to bind to a unique surface area of HLA-B*57:01 that is capable of inducing TCR recognition. However, the two residues that distinguish ABC-sensitive HLA-B*57:01 from ABC-insensitive HLA-B*57:03 are located at the bottom of the HLA-binding groove and are unlikely to contact the T-cell receptor (Illing *et al.*, 2012). Finally, the danger model, also does not explain its HLA restriction. In the last trimester, several authors have provided evidence that ABC is able to bind to HLA-B*5701 and change its structure/specificity, thereby influencing the repertoire of self-peptide ligands to be presented (Chessman *et al.*, 2008; Yang *et al.*, 2009; Bharadwaj *et al.*, 2011; Illing *et al.*, 2012; Norcross *et al.*, 2012a; Ostrov *et al.*, 2012). The authors found specific self-peptides, which were presented to HLA-B*57:01 only in the presence of ABC, and recognized by T cells of hypersensitive patients (Ostrov *et al.*, 2012). Specifically, Illing *et al.* (2012) reveal that the binding of ABC to HLA-B*57:01 alters the F pocket's binding preference for side chains of the amino acids. The authors observed no such change in the peptides that bound to HLA-B*57:03, confirming the specificity of ABC-induced HSR to HLA-B*57:01. These evidences refute the hypothesis that ABC

adducts can act as haptens and trigger an immune response, but does not exclude it. HSR may be not restricted to one mechanism. The predictive test for HLA-B*57:01 has a negative predictive value of 100% and, as recommended in the clinical practice, all patients on ABC included in the present work were negatives and therefore is not expected to develop such reactions.

The molecular mechanisms of ABC-induced cardiotoxicity are even less understood than those of HSR. Although an immune-mediated response can be envisioned, metabolic activation of ABC to aldehyde intermediates might play a significant role in this context. This hypothesis is consistent with the fact that several aldehydes and its ability to site-specific protein modification have been associated with several mechanism of cardiotoxicity, as cardiac overexpression of ADH (Guo and Ren, 2010b), lipid peroxidation, mitochondrial electron transport, interference on calcium homeostasis (Josephson *et al.*, 1991), in the regulation of inflammation, apoptosis, and other cellular signaling (Leonarduzzi *et al.*, 2004).

A well-known example of cardiotoxicity through aldehydes is the ecstasy. Its metabolites are related with the production of reactive oxygen species and with the formation of GSH adducts, which can lead to GHS depletion (Carvalho *et al.*, 2004). ABC-conjugated aldehydes may be related with these mechanisms, through mitochondrial damage, apoptosis or through protein modification, causing depletion in protein function and, therefore, leading to myocardial dysfunction, tachycardia, cardiac ischemia and heart failure. While more research is needed to clarify the mechanisms underlying ABC-induced cardiotoxicity there is a plausible role for this ABC *Edman* adduct as biomarker of ABC-induced cardiotoxicity, which could be an excellent clinical tool to prevent heart injury on those patients on long term ABC use.

Until now there was no experimental evidence that ABC could be bioactivated to a metabolite subsisting long enough in humans to undergo protein modification, but in the present study it was proved that the conjugated aldehyde formed by ABC metabolism does have that capability. However, presenting this evidence does not imply support for an "exclusively-hapten" hypothesis, nor definitely explains cardiotoxicity. Clearly more evidence is needed to clarify the mechanism(s) of ABC-induced HSR and cardiotoxicity, but summing up, the results reported herein demonstrate that the search for causal relationships between the formation of ABC-derived protein adducts and the occurrence of ABC-induced toxic events in patients is worth pursuing. Moreover, methodology described in the present study, for adduct identification and quantification, is simple, sensitive, accurate, reliable, and applicable to high throughput analyses, as required for testing clinical samples. Moreover, this approach allowed trapping ABC-conjugated aldehyde *in vivo*, a significant accomplishment since until today it had not been achieved. As such, its biomonitoring is of unquestionable worth for further ABC clinical toxicological studies. In the future, the sample size will be enlarged, and the factors that may influence ABC-*Edman* adducts formation (as ADH polymorphisms, time of exposition of ABC and the other already mentioned herein) will be evaluated. The adduct concentration will also be related to markers of heart function (routine cardiac imaging studies and blood biomarkers) aiming at clarifying the adduct role as cardiotoxicity biomarkers.

The present study is part of a translational investigation project, which involved different areas of knowledge, in a team composed by Clinicians, Pharmacologists and Chemists. My integration into this multidisciplinar and deeply committed team, allowed me to contact with different backgrounds from my base formation, allowing not only an enormous professional gain but also personal.

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Annex I

Case Report Form

FACULDADE DE CIÊNCIAS MÉDICAS | UNIVERSIDADE NOVA DE LISBOA

INFORMAÇÃO DO HOSPITAL

Médico | Telefone

Hospital | E-mail

CRITÉRIOS DE INCLUSÃO E EXCLUSÃO

	Não	Sim
≥ 18 anos de idade	☐	☐
Consentimento informado	☐	☐
Suspeita de não adesão *Adesão - cumprir pelo menos 95% da terapêutica antiretroviral*	☐	☐

INFORMAÇÃO DO DOENTE

Doente (n) |___|___|___|___|___|___|___|___|

Data de nascimento |DD|MM|AA|

Sexo F ☐ M ☐

Peso (Kg) |___|___| Altura (*cm*) |___|___|___|

Etnia: Caucasiana ☐ Negra ☐ Outra

Subtipo HIV: _____

Consumo de álcool N ☐ S ☐ ESPECIFICAR

Uso de drogas injectáveis N ☐ S ☐ ESPECIFICAR Fumador(a) N ☐ Y ESPECIFICAR

Reacções adversas N ☐ S ☐ VER ANEXO I

Patologias associadas N ☐ HCV ☐ HBV ☐ Diabetes ☐ Outra ☐ ESPECIFICAR

Infecções oportunistas N ☐ Tuberculose ☐ Outra ☐ ESPECIFICAR

Primeira terapêutica antiretroviral N ☐ S ☐

Por favor, enviar o formulário completo acompanhado das amostras para:

Departamento de Farmacologia, Faculdade de Ciências Médicas
Campo dos Mártires da Pátria, 130, 1169-056 Lisboa
Tel.: 218 803 035 **Fax**: 218 803 083 **E-mail:** farmacologia@fcm.unl.pt, sofia.pereira@fcm.unl.pt
telemóvel Sofia Pereira 96 4243174

Terapêuticas antiretrovirais anteriores (data de início/fármaco)	|___|___| |___|___| |___|___|		
	|___|___| |___|___| |___|___|		
	|___|___| |___|___| |___|___|		
	|___|___| |___|___| |___|___|		
	|___|___| |___|___| |___|___|		
Abacavir	Data de início *(dia/mês/ano)*	mg por toma	Nº de tomas
	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
Co-terapêutica antiretroviral	Data de início *(dia/mês/ano)*	mg por toma	Nº de tomas
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
Outros fármacos Não ☐ Sim ☐ (incluir todos os que tomou no último mês) (incluir fitoterápicos, medicamentos de venda livre, etc.)			
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|
NOME DO FÁRMACO	|___|___| |___|___| |___|___|	|___|___|___|___|	|___|

Parâmetro	Valor	Unidade 1	Unidade 2	Data

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Departamento de Farmacologia, Faculdade de Ciências Médicas
Campo dos Mártires da Pátria, 130, 1169-056 Lisboa
Tel.: 218 803 035 **Fax**: 218 803 083 **E-mail:** farmacologia@fcm.unl.pt, sofia.pereira@fcm.unl.pt
telemóvel Sofia Pereira 96 4243174

Subpopulações de CD_4	|___|___|___|___|___|___|	☐ *cel/mm³*	☐ _____	|DD|MM|AA|
Subpopulações de CD_8	|___|___|___|___|___|___|	☐ *cel/mm³*	☐ _____	|DD|MM|AA|
Carga viral	|___|___|___|___|___|___|	☐ *cópias/mL*	☐ _____	|DD|MM|AA|
Hemoglobina	|___|___|___|___|___|___|	☐ *g/dL*	☐ _____	|DD|MM|AA|
Plaquetas	|___|___|___|___|___|___|	☐ *cel/mm³*	☐ _____	|DD|MM|AA|
Leucócitos	|___|___|___|___|___|___|	☐ *g/L*	☐ _____	|DD|MM|AA|
Neutrófilos	|___|___|___|___|___|___|	☐ *cel/mm³*	☐ _____	|DD|MM|AA|
Linfócitos	|___|___|___|___|___|___|	☐ *cel/mm³*	☐ _____	|DD|MM|AA|
Monócitos	|___|___|___|___|___|___|	☐ *cel/mm³*	☐ _____	|DD|MM|AA|
Tempo de protrombina (INR)	|___|___|___|___|___|___|	☐ *%*	☐ _____	|DD|MM|AA|
α_1-glicoproteína ácida	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Glicémia	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Creatinina	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Ureia	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Albumina	|___|___|___|___|___|___|	☐ *g/L*	☐ _____	|DD|MM|AA|
AST	|___|___|___|___|___|___|	☐ *U/L*	☐ _____	|DD|MM|AA|
ALT	|___|___|___|___|___|___|	☐ *U/L*	☐ _____	|DD|MM|AA|
γ–GT	|___|___|___|___|___|___|	☐ *U/L*	☐ _____	|DD|MM|AA|
Fosfatase alcalina	|___|___|___|___|___|___|	☐ *U/L*	☐ _____	|DD|MM|AA|

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LDH	|___|___|___|___|___|___|	☐ *U/L*	☐ _____	|DD|MM|AA|
Bilirrubina total	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Bilirrubina directa	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
PCR	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Ácido hialurónico	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Haptoglobina	|___|___|___|___|___|___|	☐ *µg/dL*	☐ _____	|DD|MM|AA|
Alfa2-macroglobulina	|___|___|___|___|___|___|	☐ *g/dL*	☐ _____	|DD|MM|AA|
Apolipoproteina A1	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Colesterol total	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
LDL – col	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
HDL - col	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Triglicéridos	|___|___|___|___|___|___|	☐ *mg/dL*	☐ _____	|DD|MM|AA|
Transferrina	|___|___|___|___|___|___|	☐ *g/L*	☐ _____	|DD|MM|AA|
Ferro	|___|___|___|___|___|___|	☐ *µg/dL*	☐ _____	|DD|MM|AA|
Sódio	|___|___|___|___|___|___|	☐ *mmol/L*	☐ _____	|DD|MM|AA|
Potássio	|___|___|___|___|___|___|	☐ *mmol/L*	☐ _____	|DD|MM|AA|
MPKA	|___|___|___|___|___|___|	☐	☐ _____	|DD|MM|AA|
CPK	|___|___|___|___|___|___|	☐ *U/L*	☐ _____	|DD|MM|AA|
História Familiar Não ☐ Sim ☐	*Notas*			

COLHEITA

Por favor, enviar o formulário completo acompanhado das amostras para:

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Campo dos Mártires da Pátria, 130, 1169-056 Lisboa
Tel.: 218 803 035 **Fax**: 218 803 083 **E-mail:** farmacologia@fcm.unl.pt, sofia.pereira@fcm.unl.pt
telemóvel Sofia Pereira 96 4243174

Antiretrovirais	Dose (*mg*)	Frequência da dose	Data de início	Hora da colheita *(24 horas)*	Hora última dose tomada *(24 horas)*	Ingestão de alimentos com a toma
Abacavir		OD ☐ BD ☐	|DD|MM|YY|	|__|__|:|__|__|	|__|__|:|__|__|	S ☐ N ☐
Fármaco1		OD ☐ BD ☐	|DD|MM|YY|	|__|__|:|__|__|	|__|__|:|__|__|	S ☐ N ☐
Fármaco2		OD ☐ BD ☐	|DD|MM|YY|	|__|__|:|__|__|	|__|__|:|__|__|	S ☐ N ☐
Fármaco3		OD ☐ BD ☐	|DD|MM|YY|	|__|__|:|__|__|	|__|__|:|__|__|	S ☐ N ☐
Comentários						

Data da colheita |DD|MM|YY|

Responsável pela colheita_____.

Responsável pela recolha de dados_____.

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